ABSTRACT

Title of Document: CHARACTERIZATION OF THE BACTERIAL COMMUNITIES ASSOCIATED WITH THE TROPICAL SACOGLOSSAN MOLLUSKS *ELYSIA RUFESCENS* AND *ELYSIA CRISPATA*.

Jeanette Davis, Doctor of Philosophy, 2015

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Mollusks are the largest group of marine invertebrates and are known to harbor bacterial communities; however, the characterization and metabolic roles of these communities to the biology of mollusks are unknown. Sacoglossan sea slugs are herbivorous mollusks well known for their unique ability among metazoans to sequester functional chloroplasts from their algal food through a process called kleptoplasty, enabling a few species of these slugs to photosynthesize. Sacoglossan mollusks are also known to sequester chemical compounds from their algal diets through a process called kleptochemistry and use these compounds as defense molecules. These defense molecules often display medicinal properties. The mechanisms for such phenomena are unknown. I characterized the bacterial communities associated with the Hawaiian sea slug *Elysia rufescens* and its algal diet

Bryopsis sp., in which the promising anticancer compound, Kahalalide F (KF) was extracted, through both culture-based and molecular analysis. I cultured a total of 460 bacteria from the mollusk and *Bryopsis* and screened them for KF production. I found a diverse assemblage of bacteria associated with this sacoglossan comprising 16 different bacterial phyla. In addition, a photosynthetic sacoglossan slug, *Elysia crispata* from two Caribbean locations and their associated alga bacterial communities were characterized. I discovered less bacterial diversity associated with this sacoglossan and found that the bacterial communities associated with *E. crispata* from different locations are more similar to each other than the bacterial communities of the associated alga. This work forms the basis for describing the bacterial community of the sacoglossans *E. rufescens* and *E. crispata* and furthers our understanding of the potential roles bacteria may play in the unusual sacoglossan niche.

CHARACTERIZATION OF THE BACTERIAL COMMUNITIES ASSOCIATED WITH THE TROPICAL SACOGLOSSAN MOLLUSKS *ELYSIA RUFESCENS* AND *ELYSIA CRISPATA*.

By

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I dedicate my dissertation to my nephews and nieces Ricky, Mekhi, Joseph, Ki-Jana, Sequoiah, Noble, Samyah, Rahim and Sabeerah for their constant hugs and funny moments which kept me motivated throughout my graduate studies.

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Statement of Contribution

Drs. Eduardo Esquenazi and Pieter C. Dorrestein performed the MALDI imaging analysis of *E. rufescens*. Bin Wang and Amanda Waters performed the KF analysis on material sent to the Hamann Laboratory through LCMS. Dr. Mohamed Donia performed metagenomics on the *Bryopsis* sp. samples.

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Chapter 1: Introduction

1.1 Rational for studying bacterial symbionts of sacoglossan mollusks

A eukaryotic genome has generally been regarded as an autonomous component that directs the organism in which it is located. Through scientific advances, we now understand that individual animals do live alone but form relationships with microbes that influence the development, function, and behavior of the animal. Recognition of the intimate relationship between hosts and their microbial associates has inspired a new theory of evolution and use of the ecological term "holobiont" meaning a host together with the associated microorganisms (Rosenberg *et al*., 2007; Bosch and McFall-Ngai, 2011). Therefore to study any animal, it is important to also consider the microbial community associated with that animal, which influences the ecology of the animal.

The relationship between a host and its associated microbes is described as a "symbiosis" a term which biologists often use in the sense of mutualism where living together benefits all partners in the relationship (Hoffmeister and Martin, 2003). However, I use the term symbiosis in the sense as originally defined by Anton deBary to mean "two dissimilar organisms living together" (1879), therefore the benefit or harm to either partner is not considered.

There are many examples in the marine environment of symbiotic microbes influencing the ecology of marine invertebrates. The shrimp *Palaemon macrodactylus* protects its embryonic surface from fungal pathogens by recruiting the bacterial symbiont *Alteromonas* sp. that produces an anti-fungal compound (Gil-Turnes *et al*., 1989). The bacterium *Pseudomonas aeruginosa* is a pathogen of the marine alga *Delisea pulchra* (Hentzer *et al*., 2003). Bacteria associated with coral

tissue and its mucus have been well studied (Ritchie, 2006; Bourne *et al*., 2008; Sharp *et al*., 2010) and research demonstrates that the healthy, normal microbial community members of the surface mucus layer protect corals from diseases by preventing the invasion and overgrowth of pathogenic bacteria (Mao-Jones *et al*., 2010). Microbes associated with marine sponges are also extensively studied and research shows microbes can play a key role in supplying nutrients to the host (Fan *et al*., 2014; Mohamed *et al*., 2008; Mohamed *et al*., 2010), regulating biofilm production (Zan *et al*., 2012), and producing chemical compounds that may be important for the host and used for therapeutics (Hill, 2004). Some of these bacteria are so important that they are even harbored in sponges that are phylogenetically and geographically distant (Montalvo and Hill, 2013).

The above examples illustrate how microbes live in association with marine invertebrates and help facilitate the ecology of the hosts. Sacoglossan mollusks are unusual marine invertebrates that possess unique features that are absent among animals; however, there are few studies of the microbes associated with these organisms and the potential roles of these microbes in sacoglossan ecology. The growing knowledge of the importance of microbial symbionts and the limited research available on the microbial symbionts of sacoglossans makes them an attractive organism in which to study bacterial symbiosis.

1.2 Sacoglossan feeding and body morphology

Sacoglossans are a unique clade of sea slugs and sea snails that belong to a diverse group of specialized marine mollusks called Opisthobranchia and are best

known for their unusual relationship with marine algae. Opisthobranch mollusks include bubble snails, headshield slugs, anaspidean sea hares, sea butterflies, sacoglossans and many nudibranchs (Göbbeler and Klussmann-Kolb, 2011). Although most animals in this group are generally referred to as sea slugs or sea snails, diverse feeding niches permitted by the development of morphological structures for dietary specialization is considered a "driving force" of Opisthobranch evolution (Kay, 1968; Thompson, 1976; Mikkelsen, 2002). Sacoglossans are distinguished from related slugs by the radula, a feeding apparatus found in all groups of mollusks except bivalves (Jensen, 1997). The morphology of the radula is linked to diet and typically is used to scrape algae from a surface or to feed directly on macroalgal tissue (Jensen, 1994). In a complete deviation from other mollusks, sacoglossan slugs form a single row of teeth on the radula with a distinct arrangement (Steneck and Watling, 1982; Jensen, 1997). This nonconformity has led to extreme stenophagy and for this reason sacoglossans are often referred to as "sap-sucking" sea slugs.

Sacoglossan slugs generally are suctorial herbivores that pierce the cell wall of filamentous algae and suck out the cell contents into their digestive system (Pierce and Curtis, 2012), rather than just rasping at the algal tissue. However, there are a few oophagous species of sacoglossans that feed on the eggs of other opisthobranchs, diatoms or seagrasses (Jensen, 1993; 1996; 1997). These slug specialists exhibit a relationship between the radular tooth shape and cell wall of algal foods (Jensen 1993, Maeda 2012). Typically the algae eaten by sacoglossans have either xylan, mannan or cellulose as the structural wall component (Jensen, 1993). Primitive

radular teeth are associated with *Caulpera* or calcified algae, in which xylan is the major structural polysaccharide. From this, blade-shaped teeth evolved which are present in species that feed on a variety of diets. Sabot-shaped teeth are the most advanced and are associated with diets of Siphonocladales or Cladophorales, which have cellulose and crossed-fibrillar textured cell walls (Jensen, 1993; Jensen, 1997). Observations on the sectorial pharynx and the arrangement of radular teeth reveal various ways to penetrate the cell wall of specific algal foods (Jensen 1993; 1997) and thus the close associations between sacoglossan slugs and their food plants have often raised the question of co-evolution (Clark and Busacca, 1978).

Sacoglossans display a diverse external morphology. In the shelled or snail clade, a large coiled shell is present in primitive slugs, tiny shells are present in more recently evolved members, and one group possess a peculiar bivalve shell. The shell-less or slug clade possesses members that have cylindrical cerata on the dorsal surface and members that have parapodia, commonly referred to as wings. Sacoglossans have cephalic tentacles that are used for sensing in their environment and most are green in color due to the ingestion of algae content.

1.3 Sacoglossan distribution, species diversity, and phylogeny

Sacoglossans have a fairly wide distribution and are found in the majority of shallow tropical and temperate marine environments (Cuz *et al*. 2013). Since these slugs are dietary specialists, they are restricted to the photic zone in habitats where their algal diets are found (Jensen, 2006). There are about 250-300 valid species of sacoglossan slugs, with new species still being described and synonymized (Jensen,

2007). The highest diversity of sacoglossans is found in the islands of the Central Pacific, including the Indo-Polynesian region with approximately 133 known species. The Caribbean islands are another center of species diversity with an estimate of 49 known species (Jensen, 2007). The number of species decreases with increasing latitude and it is believed that sacoglossans in cold waters are mostly eurythermic warm water species.

Sacoglossan mollusks consist of two clades, the shelled Oxynoacea and the shell-less Plakobranchacea (Christa, 2014) (Figure 1.1). The shelled slugs are divided into three families, represent around 20% of sacoglossans and are commonly referred to as sea snails. The family Juliidae is unusual in that the shells consist of two hinged valves and look nearly identical to those of a clam as opposed to a typical shelled snail. These snails represent the oldest members of sacoglossans and are thought to have appeared during the early Eocene era (Jensen 2006) when gastropods underwent great diversification. Initially these sacoglossans were only known from shell fossils and were considered bivalves until the first live specimen was discovered feeding on *Caulerpa* sp. (Kawaguti and Baba, 1959). Since this discovery, several genera of these extraordinary sacoglossans have been found in different locations feeding and living in association with several *Caulerpa* spp. The shelled sacoglossans are thought to be primitive and feed exclusively on algae of the genus *Caulerpa* (Thompson, 1984; Jensen 1997). The shell-less clade represent the majority of sacoglossans and are commonly referred to as sea slugs; however, most members in this group do have a shell during the larval stage after hatching (Rumpho, 2001). The sea slug clade is further divided into the ceras-bearing slugs, which resemble nudibranchs, in the

family Limapontioidea and the parapodia-bearing slugs in the family Plakobranchoidea (Figure 1.1). The shell-less Plakobranchacea have a wider food spectrum compared to shelled Oxynoacea, with the genera *Elysia* showing the widest range of diets. Members of this genus can feed on a range of siphonaceous green algae and sometimes red algae.

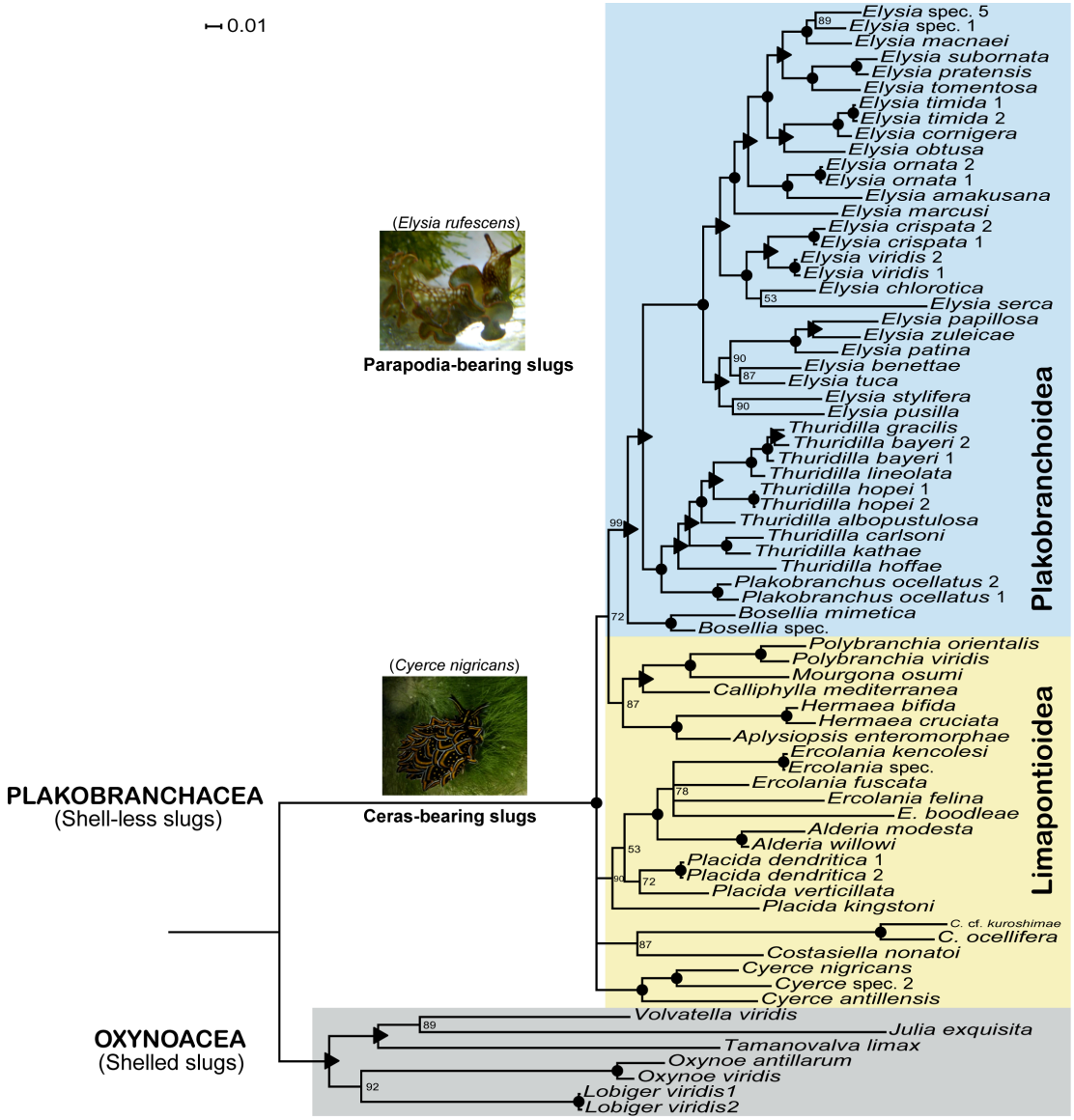


Figure 1.1. Phylogeny of Sacoglossa. Adopted from Händeler *et al*. (2009) with permission.

1.4 Sacoglossan kleptoplasty

The observation of green pigments as small plant cells in sacoglossans was first made many years ago (de Negri and de Negi, 1876; Brandt 1883) and was first noted in the sacoglossan *Caliphylla mediterranea* (Costa 1867). However, it wasn't until nearly a century after the initial observation that electron microscopy revealed that the green pigments are plastids stored in the digestive cells of *Elysia atroviridis* (Kawaguti and Yasmasu, 1965). The process by which intact plastids, notably chloroplasts from ingestion of food algae are incorporated into the host organism's cell is referred to as kleptoplasty (Clark, 1990). The sequestered chloroplast is called a "kleptoplast" (Waugh and Clark, 1986) and is harbored intracellularly in cells of the digestive diverticula, enabling photosynthesis in some species of sacoglossans (Pierce and Curtis, 2012). These kleptoplasts must be acquired in each generation and are not passed on to progeny (Williams and Walker, 1999; Händeler and Wägele, 2007; Rumpho *et al*., 2011). Kleptoplasty is a feature exhibited in members of the Protista kingdom including Foraminifera, Dinoflagellates, and Ciliates (Bernhard and Bowser, 1999; Gast *et al*., 2007; McManus *et al*., 2012;). Sacoglossans are the only members of the animal kingdom that display kleptoplasty (Clark *et al*., 1990; Rumpho *et al*., 2000, Händeler *et al.,* 2009). This association has been extensively studied using several different methods.

Kleptoplasty varies extensively in duration and function amongst sacoglossan slugs, with some slugs immediately digesting chloroplasts, while others maintain functional plastids for months in the absence of their algal diets (Pierce and Curtis, 2012; Rumpho *et al*., 2001). It was initially believed that although a variety of

sacoglossans could harbor chloroplasts, longer duration of kleptoplasty was restricted to the family Elysiidae that fed on Siphonales algae (Marín and Ros, 2004). Clark *et al*. disproved this hypothesis by measuring carbon fixation in a variety of slugs and suggested several levels of kleptoplasty that could be categorized into non-functional kleptoplasty and functional kleptoplasty (1990). Non-functional kleptoplasty include the uptake of plastids that are immediately degraded or structurally maintained for up to a few days without detectable photosynthetic function. Functional kleptoplasty implies active photosynthesis from the uptake of plastids and can be further categorized as short-term retention (StR) and long-term retention (LtR). Short-term retention kleptoplasty allows for photosynthesis for up to 2 weeks whereas LtR kleptoplasty permits photosynthesis up to several months after the subsequent starvation (Clark *et al*., 1990; Händeler *et al.,* 2009).

There are only six known species of sacoglossan that exhibit LtR kleptoplasty, *Elysia timida*, *Elysia crispata*, *Elysia virdis*, *Elysia chlorotica*, *Elysia clarki*, and *Plakobranchus ocellatus* (Pierce and Clark, 2012; Händeler *et al.,* 2010; de Vries *et al*., 2013). Of these species, *E. chlorotica* exhibits the longest LtR kleptoplasty, exclusively feeding and acquiring plastids from only two *Vaucheria* species. This is a specific and obligate sacoglossan-algal association and *E. chlorotica* will not complete metamorphosis and develop into adult without the presence of its algal diet. Once development has occurred in this sacoglossan, the original acquired plastids can sustain starved slugs for their entire lifespan, up to 10 months, through photoautotropy (Rumpho, 2011).

The wide variation in sacoglossan kleptoplasty is intriguing. Researchers suggest that the initial uptake and storage of chloroplasts serves as a camouflaging mechanism as the ingested cells cause green coloration of the animals (Wägele and Klussmann-Kolb, 2005). This short-term storage of chloroplasts may have led to photosynthesis and sacoglossans that could retain functional chloroplasts would have the competitive advantage of surviving when algal food was scarce and therefore would have enhanced fitness. Researchers have also assessed kleptoplasty variation coupled with phylogeny and their work supports that: 1) kleptoplasty was acquired at the basal position of Sacoglossa as non-functional, 2) StR kleptoplasty was gained in a common ancestor Plakobranchoidea, and 3) LtR kleptoplasty evolved independently in specific sacoglossan species. Morphology of the LtR slugs is thought to be important in plastid retention, as the wing-like parapodia in these animals are able to unfurl or retract to control light exposure (Wägele and Klussmann-Kolb, 2005; Maeda *et al*. 2010). These studies suggest the evolutionary drift from non-functional to functional kleptoplasty in sacoglossans. Variations in kleptoplasty may provide a unique opportunity to investigate the ongoing evolutionary process in the association between animal cells and functional chloroplasts.

1.5 Sacoglossan kleptochemistry

In addition to sequestering the plastids from algae, many members of sacoglossan slugs are able to acquire secondary metabolites from their algal food (Paul and Van Alstyne, 1988; Cimino *et al*., 1990). With a few exceptions, sacoglossans feed on siphonalean green algae (Jensen, 1997) that are known to produce various noxious

compounds, which serve as feeding deterrents of generalist herbivores (Paul and Fenical, 1986; Hay *et al*., 1989). Nevertheless, sacoglossans herbivores have developed a tolerance for these compounds. The process by which sea slugs synthesize chemical precursors or incorporate chemicals from their plant diet is termed kleptochemistry (Avila, 1995). Sacoglossan slugs may sequester, concentrate or modify dietary metabolites that are often released in the mucous secretion as defensive molecules (Cimino and Ghiselin, 1998; Cimino *et al*., 1999; Marín and Ros, 2004). Cimino and Ghiselin investigated the chemistry of sacoglossans and suggested an intriguing relationship between chemical defense and evolution (1998). Their research suggests reduction of the shell correlates with the advancement of defensive strategies including dietary derived metabolites.

Chemical metabolites have been detected in several sacoglossan-algal associations. One classic example is *Caulerpa* spp. that are chemically rich macroalgae found worldwide, commonly in shallow tropical and subtropical marine habitats (Paul *et al*., 2007). Various toxic terpenes, including caulerpenyne, have been isolated from species of *Caulpera* (Paul and Fenical 1986; Dumay *et al*., 2002). Caulerpenynes are sesquiterpenoid, which consist of three isoprene units ($C_{15}H_{24}$). These compounds are biosynthesized through the methyl-erythritol-4-phosphate (MEP) pathway that occurs in the chloroplasts where CO2 is fixed during photosynthesis (Pohnert and Jung, 2003). Caulerpenyne compounds display a variety of biological roles including wound healing in the alga, can induce apoptosis in mammalian cells, and show neurological effects on invertebrate model organisms (Mozzachiodi *et al*., 2001; Adolph *et al*., 2005; Cavas *et al*., 2006).

Caulerpa spp. were among the first green algae studied by natural product chemists and it is hypothesized that the associated chemical metabolites facilitate its biological invasiveness, as most herbivorous fish and invertebrates avoid these algae (Blackman and Wells, 1976; Paul *et al*., 2007). However, shelled and shell-less sacoglossans species of *Elysia*, *Oxynoe*, *Lobiger* and *Volvatella* feed on various *Caulerpa* spp. in which *Caulerpa*-derived compounds have been detected and shown to deter predators (Jensen, 1983; Marín and Ros, 2004; Davis *et al*. 2005). In sacoglossans, these *Caulerpa*-dervied compounds are unpatatable and can affect the physiology of fish predators by altering enzymatic detoxification systems in their livers (Uchimura *et al*., 1999; Paul *et al*., 2007). In addition to their ecological roles *Caulerpa*-derived compounds show antimicrobial and anti-proliferative activities, which are of potential importance for natural products discovery (Paul *et al*., 2007). Metabolites similar in structure to caulerpenyne have been extracted from related green algae of the genera *Udotea* and *Halimeda* and found in sacoglossan members that feed on these algae (Paul and Fenical, 1986). Specifically, *Elysia halimedae* of Guam feeds on *Halimeda macroloba* and sequesters the algal-derived diterpenoid which has shown to be a feeding deterrent secreted in the mucus when this slug is disturbed and is present in egg masses of the slugs (Paul and Van Alstyne, 1988). The sacoglossans *Cyerce nigricans* and *Elysia* sp. from the Great Barrier Reef, Australia feed on the green alga *Chlorodesmis fastigiata* and sequester the diterpenoid chlorodesmin which have been shown to deter reef fish (Hay *et al*., 1989). All of these sacoglossan-algal associations illustrate the ability of sacoglossans to

acquire specific algal metabolites, however some slugs have developed additional mechanisms for obtaining algal-derived chemical metabolites.

Sacoglossans within the genus *Elysia*, are able to either accumulate sesquiterpenoids, diterpenoids and depsispetides from their algal diets and then, modify such compounds, or biosynthesize de novo polypropionates (Gavagnin *et al*., 1994; Ciavatta *et al*., 2006). Therefore many sea slugs of this genus are capable of harboring multiple metabolites. The ability of sacoglossan species to convert typical *Caulerpa*-derived metabolites into more toxic compounds (Di Marzo *et al*. 1991; Gavagnin *et al*., 1994a) using specific enzymes found in selective slugs (Pawlik, 1993) is well documented. Sacoglossans that are capable of synthesizing de novo metabolites may produce more potent feeding deterrents than some of the algal metabolites; this is evident in slug species that feed on algae that are not apparently chemically defended (Ireland and Faulkner, 1981; Gavagnin *et al*., 1994b). Many of these metabolites are polypropionates and are also found as defensive compounds in other herbivorous and carnivorous opisthobranchs (Avila, 1992). Thus the metabolic pathways synthesizing polypropionates may be plesiomoporhic within opisthobranchs (Jensen, 1997). Gavagnin *et al*. assessed chemical strategies adopted by Mediterranean and Caribbean sacoglossans to obtain their secondary metabolites and found that various *Elysia* spp. have two different metabolic pathways to produce distinctive compounds and the preferred pathway is often linked to the presence or absence of the algal diet and/or location (2000).

The Hawaiian *Elysia* species and the alga *Bryopsis* sp. serve as an added example of sacoglossan slugs displaying distinctive compounds acquired from their algal diet.

The kahalalides are a family of structurally distinct depsipeptides isolated from the sacoglassans *Elysia rufescens*, *Elysia ornata* or *Elysia grandifolia* and their preferable algal diet *Bryopsis* sp. A total of 24 kahalalide peptides have been elucidated from this sacoglossan-algal association and have a wide range of size and structure (Gao and Hamann, 2011). Of these peptides, kahalalide F (KF) (Figure 1.2) was the first peptide discovered and acts as a chemical defense molecule in both *E. rufescens* and *Bryopsis* sp. (Hamann and Scheuer, 1993). In addition to its role in chemical defense, KF has been evaluated in phase II clinical trials in hepatocellular carcinoma, non-small-cell lung cancer, and melanoma (Hamann, 2004).

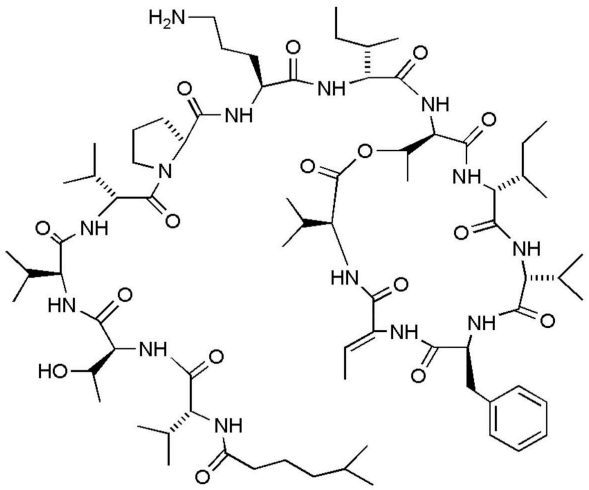


Figure 1.2. Chemical structure of kahalalide F (KF). From Jimeno *et al*. (2006) with permission.

1.5.1 Kahalalide F: mechanism of action

The mechanism of action of KF has not yet been completely characterized (Gao and Hamann, 2011). However many targets have been identified that suggest that KF exhibits a novel mechanisms of action. The plasma membrane is thought to be the first target of KF. Human prostate and breast cancer cells treated with KF display breakage in the plasma membrane, which allows for changes in osmotic balance, permeabilization and cell alterations (Janmaat *et al*., 2005; Gao and Hamann, 2011). KF has been hypothesized to act similar to cytotoxic ionophores that induce cell death by forming new ion channels in the membrance and/or changing the activity of existing channels (García-Rocha *et al*., 1996; Gao and Hamann, 2011). The lysosomes are thought to be another target for KF action as the lysosomes of cancer cells treated with KF become dramtically swollen and the lysosomal pH increases (García-Rocha *et al*., 1996). Most anticancer drugs are thought to induce apoptosis that is characterized by morphological changes including cell shrinkage, cytoplasmic condensation, ladder DNA degradation, and nuclear fragmentation resulting in an active programmed cell death (Janmaat *et al*., 2005). KF exhibits the opposite morphological changes such as cell swelling, degradation of crucial organelles such as lysosomes and mitochondria, and no DNA damage leading to a passive cell death relating to physical or chemical injury. These cell features are typical of a process called oncosis. The alterations in cell morphology and several biochemical results support the induction of KF by oncosis as opposed to apoptosis (Gao and Hamann, 2011) and is one unique feature of KF.

Another novel feature of KF is the ability to inhibit receptor tyrosine kinase ErbB3 (HER3) and the phosphatidylinositol 3-kinase-Akt signaling pathways in breast cancer cells (Janmaat *et al*., 2005; Sewell *et al*., 2005). This kinase and pathway are directly linked to proliferation, chemotherapeutic resistance, and the promotion of metstasis (Holbro *et al*., 2003; Wang *et al*., 2010). This further suggests KF is involved an unknown oncosis signaling pathway. KF currently represents the first anticancer compound that can inhibit ErbB3 receptors.

The novel mode of action of KF has led to persistent clinical trials under the leadership of the company PharmaMar. Phase clinical trials of KF have lasted for nearly a decade (Luber-Narod *et al*., 2000; Gao and Hamann, 2011). Elisidepsin (Irvalec®, PM02734), a synthetic peptide derived from KF has been evaluated in phase I/II clinical trials (Coronada *et al*. 2010). Elisidepsin shows potent and broad cytotoxicity effects on various cancer cells at concentrations ranging from 0.4 to 2 μM, which are relevant concentrations for clinical trials (Serova *et al*., 2013). Unfortunately, in Spring 2012 PharmaMar decided to suspend development of Irvalec and concentrate on advancing other marine-derived products.

1.6 Roles of bacteria associated with marine mollusks

Sacoglossan mollusks have developed intriguing mechanisms to aid in the fitness of their lifestyle by acquiring both chloroplast and chemicals from their algal diets. It is still unclear how chloroplasts are maintained in sea slugs or how algal toxins that generally deter predators can accumulate in sacoglossans. However, the importance of bacteria to the overall ecology of marine invertebrates is becoming increasingly

appreciated with new sequencing technology. The unusual mechanism displayed in sacoglossans make them attractive model systems in which to study bacterial symbiosis.

Many mollusks live in seawater habitats where bacteria are extremely abundant achieving densities up to 10^6 cells per ml of seawater (Azam, 1998). Mollusks do not have known acquired immunological memory (Sminia and Van der Knapp, 1986; Hopper *et al.,* 2007). These factors thus suggest that mollusks may have developed defense strategies to protect themselves from exposure to a high density of microorganisms, including potential pathogens. The use of secondary metabolites is well documented in the phylum *Mollusca* as part of their communication systems (Cimino *et al*., 1991; Zatylny *et al*., 2000; Cummins *et al*., 2006), predatory behavior (Craig, 2000; Kanda *et al*., 2003) and defensive secretions (Pawlik *et al*., 1986; Marín *et al*., 1991; Kelley *et al*., 2003; Derby *et al*., 2007). The abundance, diversity and chemical potential of mollusks make this phylum of interest for studies of microbial symbiosis.

Most molluscan-associated bacterial community studies highlight the class *Bivalvia*, primarily because of their economic and ecological value. Several bacterial pathogens have shown to cause motility of larval and juvenile oysters (Paillard *et al*., 2004; Karim *et al*., 2013; Elston *et al*., 2008). Some oyster pathogens can even cause human diseases if infected animals are consumed (Thompson *et al*., 2004; Pruzzo *et al*., 2005); therefore one ecological role of bacteria associated with molluscan members is an infectious agent. Bacteria can also be beneficial to the health of mollusks. Research reveals several bacterial strains prevent harmful bacteria from

thriving in mollusks and often times these bacteria can produces specific compounds that inhibit the growth of pathogenic bacteria (Kesarcodi-Watson *et al*., 2008; Kapareiko *et al*., 2011). Lokmer and Wegner (2014) assessed the hemolymph microbiome of Pacific oysters and found that bacteria present in the hemolymph of healthy oysters were significantly affected by temperature stress, but not by infections, illustrating the dynamic stability of the hemolymph microbiome. Bacteria can also be important symbionts of the digestive system and help in degrading compounds from the environment (Mayasich and Smucker, 1987). Recent deep sequencing analysis revealed a diverse and novel bacterial community associated with a putative core gut microbiome of oysters in which the functions of these bacteria are unknown (King *et al*., 2012). Many novel γ- proteobacteria have been isolated from the intestinal tract of marine gastropods including sea hares and sea slugs that are not related to sacoglossan slugs (Kurahashi and Yokota, 2004); the roles of these bacteria are also unknown. The bacterium *Vibrio fisheri* induces formation of the light organ in the *Euprymna scolopes* that aids in camouflaging during nightly feeding (Nyholm *et al*., 2000) and continues to serve as model system for bacterial symbiosis in a marine host.

Bacteria can also provide nutrients to mollusks. Research suggests that although nutrients can be acquired through filter feeding, specifically in bivalves, most adults obtain necessary nutrients from microbial symbionts (Roeselers and Newton, 2012). Sulfur-oxidizing and methanotrophic symbionts located in the ctenidia, the comblike respiratory gills of mollusks, are now recognized as a widespread nutritional strategy established in five separate families of marine bivalves that occupy habitats from

intertidal zones to hadal depths (Fisher, 1990; Cavanaugh *et al*., 2006; Dubilier *et al*., 2008; Taylor and Glover, 2010). One study characterized chemoautotrophic symbionts in two mussel populations during various life stages and found that the symbionts were present in all samples regardless of life stage. Hydrothermal vent gastropods from the same family in two separate locations harbor sulfur-oxidizing ε- and γ- proteobacteria (Urakawa *et al*., 2005). These examples illustrate that bacteria can play various important roles in the ecology of marine mollusks and there are still many unknown roles of bacterial symbionts.

About 90% of molluscan biological and chemical diversity is found in the class Gastropoda (Benkendorff, 2010) with Opisthobranchs being one of the most diverse groups of gastropods. As previously mentioned, opisthobranchs possess biological features related to foraging or defensive strategies that are unique or rare in the animal kingdom (Wägele, 2004). Sacoglossan mollusks have evolved to perform unique features with specialized structures in a microbe-rich environment; therefore, sacoglossan ecology require an examination of not just the slugs and their algal diets but the bacteria that live in association with these organisms. Kurahashi and Yokota (2007) were the first to isolate and characterize a novel γ- proteobacteria symbiont of the sacoglossan *E. ornata* collected off the coast of Japan. Recently, 16S rRNA-based metagenomics of the bacterial communities associated with the sacoglossan *E. chlorotica*, its algal prey *V. litorea* from their native environments, and *E. chlorotica* after being starved of algal prey and bred in the laboratory revealed diverse bacterial profiles that varied between populations and among all conditions except for the laboratory-bread samples (Devine *et al.*, 2012). These results provide an example of

bacteria associated with a sacoglossan, however we still have not yet uncovered the full diversity or potential roles of these bacteria.

1.7 Focus and objectives

Sacoglossans are unusual animals that sequester chemicals and chloroplasts from their algal diets, enabling some species to secrete defense molecules in their mucus and photosynthesize. These unusual mollusks have evolved in a microbe-rich environment. My research aimed to characterize the bacterial community associated with two tropical sacoglossan mollusks. To fulfill this research goal, my research objectives are:

1. Isolate and characterize bacteria associated with a chemically defended sacoglossan and its secreted mucus, *E. rufescens*.
2. Characterize the bacterial communities associated with a photosynthetic sacoglossan and its associated algae, *E. crispata*.
3. Consider the potential roles of the bacteria associated with these sacoglossans and determine if there are shared bacteria associated with these two sacoglossans.

The results presented here provide the first insights into the potential roles of bacteria in the sacoglossan-algal association. Insights are also provided into the diversity of cultivable bacteria associated with a sacoglossan. The research presented here will be useful in determining which bacteria are important for acquiring chemicals and chloroplasts from sacoglossan algal diets. In addition this work contributes to the

understanding of phylogenetic diversity of bacteria associated with sacoglossans and the growing knowledge of the vast range of animal-bacterial interactions.

Chapter 2: Characterization of the bacterial community of the chemically defended Hawaiian sacoglossan *Elysia rufescens* and its secreted mucus

2.1 Abstract

Sacoglossans are known for their ability to sequester chemicals from their algal diet through a process called kleptochemistry, enabling them to use such compounds as defense molecules in mucus secretion. The bacterial diversity associated with sacoglossans is not well understood. In this study, I coupled traditional cultivation-based methods with 454 pyrosequencing to examine the bacterial communities of the chemically defended Hawaiian sacoglossan *Elysia rufescens* and its secreted mucus. *E. rufescens* contains a defense molecule, kahalalide F, that is possibly of bacterial origin and is of interest because of its anti-fungal and anticancer properties. My results showed that there is a diverse bacterial assemblage associated with *E. rufescens* and its mucus with secreted mucus harboring higher bacterial richness than entire *E. rufescens* samples. The most abundant bacterial groups affiliated with *E. rufescens* and its mucus are *Mycoplasma* spp. and *Vibrio* spp., respectively. My analyses revealed that the *Vibrio* spp. that were highly represented in the cultivable assemblage were also abundant in the culture-independent community. Epifluorescence microscopy and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) were utilized to detect the chemical defense molecule, kahalalide F, on a longitudinal section of the sacoglossan.

2.2 Introduction

Mollusca is the largest marine phylum, with approximately 23% of all known named marine invertebrates (Benkendorff, 2010), yet there are few studies assessing bacterial symbiosis associated with members of this phylum. Microbial symbionts can provide nutrients to the host, assist in structural development, cause disease, protect the host from infectious microbes, or initiate chemical responses to the environment (Holmström and Kjelleberg, 1994; Donachie and Zdanowski, 1998; Mearns-Spragg *et al*., 1998), among other roles. There has been strong evidence suggesting the epithelium of marine *Hydra* actively selects and shapes its microbial community and that there is a direct connection between the epithelia and microbiota (Fraune and Bosch, 2007; Bosch, 2012). The zebrafish *Danio rerio* gut development is dependent on the presence of a specific core gut microbiome (Bosch and McFall-Ngai, 2011; Roeselers *et al*., 2011). In addition to impacting the hosts, marine microbial symbionts can also be valuable resources for drug discovery. In some cases the symbionts may be the true producers of compounds found in invertebrates (Schmidt et al., 2005; Piel et al., 2004; Davidson *et al*., 2001). These bioactive products often possess anticancer and antimicrobial properties (Proksch *et al*., 2002; Piel, 2004; Suarez-Jimenez *et al*., 2012).

Sacoglossans are best known their ability to incorporate and use intact chloroplasts from their algal diet (Rumpho *et al*., 2001) and sequester chemicals from their algal food (Avila, 1995). Sacoglossans are the only metazoans known to photosynthesize and the range of photosynthetic ability varies among sea slugs (Pelletreau *et al*., 2011). The herbivorous sacoglossan sea *Elysia chlorotica* retains

functional plastids in the cells lining the digestive tract for several months in the absence of the algal prey and continues to photosynthesize (Rumpho *et al*., 2000, Rumpho *et al*., 2001). One hypothesis proposed to explain how plastids continue to function within the slug cells in the absence of the algae is horizontal gene transfer (HGT) of essential photosynthetic genes from the algal diet to the sea slug genome. Pierce *et al*. analyzed the transcriptome of *E. chlorotica* and reported the presence of at least 101 chloroplast-encoded gene sequences and 111 algal transcripts, 52 of which were nuclear genes (Pierce *et al*., 2012). However, the mechanisms for long-term maintenance of photosynthesis is still unresolved as recent genomic data from the *E. chlorotica* germ line (egg DNA) and from its algal prey, *Vaucheria litorea* failed to provide evidence for alga-derived HGT into the germ line of the sea slug (Bhattacharya *et al*., 2013).

Sacoglossan slugs feed on specific algae that possess chemical compounds with antimicrobial, antifouling and feeding-deterrent abilities (Smyrniotopoulos *et al*., 2003; Dobretsov *et al*., 2006). These compounds or their modified forms can be detected in the slugs, specifically in the mucous secretion, where the compounds are thought to function in defense (Marín and Ros, 2004). The bacterial communities associated with the sacoglossan *E. chlorotica* and its algal prey *V. litorea* were recently characterized (Devine *et al*., 2012); however from our current knowledge there are no bioactive compounds that have been established to be produced by the sacoglossan or bacterial partners in this association.

The sacoglossan *Elysia rufescens* and the alga *Bryopsis* sp. is another sacoglossan-algae association and in this case the sacoglossan is chemically protected

against fish predators by the deterrent properties of kahalalide F (KF) (Becerro *et al*., 2001). KF is a promising anticancer compound that has been extracted from both *Elysia rufescens* and *Bryopsis* sp. (Hamann and Scheuer, 1993). The bioactive compounds associated with *E. rufescens* are well investigated (Hamann and Scheuer, 1993; Hamann, 2004; Gao and Hamann, 2011), and preliminary data suggested that two strains of *Vibrio* sp. isolated from *E. rufescens* produce the compound KF, with production confirmed by liquid chromatography-mass spectrometry (LCMS) and nuclear magnetic resonance (NMR) (Hill *et al*., 2003). However, recent attempts to isolate KF from cultured *Vibrio* strains have given inconsistent results and more studies are needed to unequivocally determine the organism(s) responsible for the production of KF. In this study I seek to better understand the bacteria associated with a chemically defended sacoglossan, *E. rufescens*. Since sacoglossans are known to secrete bioactive compounds in their mucus (Marín and Ros, 2004), the bacterial communities associated with *E. rufescens* mucus as well as the *E. rufescens* itself were investigated.

I used a comprehensive approach by coupling traditional cultivation with cloning and deep pyrosequencing of 16S rRNA gene amplicons to characterize and compare the bacterial communities associated with *E. rufescens* and its secreted mucus.

2.3 Experimental methods

2.3.1 Sample collection and processing

Elysia rufescens was collected by snorkel at Black Point Bay, Honolulu, HI ($21^{o}15^{'}$N, $157^{o}47^{'}$W) in March 2010. Sixteen sacoglossan individuals were placed in separate plastic collection bags filled with surrounding seawater and transported to a laboratory for processing within 1 hour of collection. Samples were rinsed 3 times with sterile 1 x PBS to remove transiently and loosely attached bacteria. Five *E. rufescens* individuals were transferred to a sterile 50 ml conical tube (Corning, Tewksbury, MA) and removed after secreting copious amounts of mucus. The secreted mucus left after the removal of the sea slugs was collected and pooled for culture-based and molecular analyses. This process was repeated for a second set of five *E. rufescens* individuals and the secreted mucus from these individuals was treated as a separate sample. One milliliter of the two separate secreted mucus samples was used for bacterial cultivation and the remaining mucus was immediately stored at -80˚C and later lyophilized (Labconco, Kansas City, MO) for molecular analysis. Three separate individuals of *E. rufescens* were used for bacterial cultivation and an additional set of three individuals were immediately stored at -80˚C and later lyophilized (Labconco, Kansas City, MO) for molecular analysis. Water samples were collected near the sea slugs in sterile 20-liter containers and approximately 5 L were filtered through three 0.22 μm pore size Sterivex-filters (Millipore, Billerica, MA) for each water sample. The Sterivex-filters were immediately stored at -20˚C for isolation of nucleic acids.

2.3.2 Bacterial cultivation and identification

Elysia rufescens was homogenized in 9 ml of sterile 1 x PBS using a mortar and a pestle. The homogenate was used to create a 10-fold dilution series of which 100 µl aliquots were plated on Marine Agar 2216 (Becton Dickinson, Sparks, MD). These plates were incubated for five days at 30˚C and morphotypes were randomly picked for identification. The same procedure was used for the secreted mucus from *E. rufescens*. DNA was extracted from the bacterial isolates obtained from *E. rufescens* and secreted mucus as described by Montalvo *et al*. (2005). Briefly, DNA from cultured representatives was extracted using the Mo Bio UltraClean Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) per manufacturer's instructions. Bacterial 16S rRNA gene fragments were PCR-amplified with eubacterial primers 27F and 1492R (Lane, 1991). Reactions were run in a PTC-200 cycling system (MJ Research, Waltham, MA) using the following cycling parameters: 300 seconds of denaturation at 94˚C, followed by 25 cycles of 30 seconds at 92˚C (denaturing), 120 seconds at 46˚C (annealing), and 90 seconds at 72˚C (elongation), with a final extension at 72˚C for 300 seconds. The PCR-products were sequenced on an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA), using 27F primer. Sequences were classified with the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier). The closest relatives for each sequence were obtained from the GenBank database using the BLASTn tool (http://blast.ncbi.nlm.nih.gov/).

2.3.3 Total genomic DNA extraction

Total genomic DNA from lyophilized *E. rufescens* and its mucus was extracted using Mo Bio PowerBiofilm DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) per manufacturer's instructions using the following modifications: vortex adaptor (Scientific Industries Inc., Bohemia, NY) for cell lysis and doubling solution BF3 to increase amplification efficiency. DNA was extracted from the Sterivex-filters obtained from seawater samples using the protocol described by Somerville *et al*. (1989).

2.3.4 Bacterial 16S rRNA gene clone library construction and identification

Bacterial 16S rRNA gene clone libraries were constructed from lyophilized *E. rufescens* and secreted mucus from *E. rufescens*. Bacterial 16S rRNA gene fragments were PCR-amplified with universal primers 27F and 1492R (Lane, 1991) and clone libraries were constructed as described by Montalvo *et al.* (2005). PCR products were gel-visualized and purified by ethanol precipitation. Purified PCR products were ligated into a pCR-XL-TOPO vector and transformed into One Shot TOP 10 chemically competent *E. coli* cells using the TOPO XL PCR cloning kit (Invitrogen Life Technologies, Carlsbad, CA). Clones were sequenced as described above.

2.3.5 Denaturing gradient gel electrophoresis (DGGE) of bacterial community

DGGE was performed on PCR amplified genomic DNA from *E. rufescens*, secreted mucus from *E. rufescens* and surrounding seawater samples using a 195 bp region corresponding to positions 341 and 534 in the 16S rRNA gene of *E. coli* using P2 and P3 primers as described by Muyzer *et al*. (1993). DGGE was performed by

using a DCode system (Bio-Rad, Hercules, CA) on an 8% (wt/vol) polyacrylamide gel with a denaturing gradient of 40 to 70% in 1 x Tris-Acetate-EDTA (TAE). Electrophoresis was performed for 16 h at 60 V at 60˚C. The gel was stained with SYBRgreen in 1 x TAE staining bath and visualized with a Typhoon 9410 image system (Amersham Biosciences, Piscataway, NJ). DGGE banding patterns were compared using BioNumerics 7.0 software (Applied Maths, Nevada) (http://www.applied-maths.com). An unweighted pair group with arithmetic mean (UPGMA) dendrogram based on the Dice similarity coefficient was generated to show the similarity in banding patterns across samples.

2.3.6 Pyrosequencing of barcoded 16S rRNA gene amplicons

Total genomic DNA was extracted as described for construction of clone libraries. Hypervariable regions V1-V3 of the 16S rRNA gene fragments were amplified with primers 27F and 534R. DNA amplification of the 16S rRNA genes was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) and 20-50 ng of template DNA in a total reaction volume of 25 µl following the Platinum *Taq* product protocol. Reactions were run in a PTC-200 cycling system (MJ Research, Waltham, MA) using the following cycling parameters: 180 seconds of denaturation at 94˚C, followed by 30 cycles of 30 seconds at 94˚C (denaturing), 30 seconds at 46˚C (annealing), and 45 seconds at 72˚C (elongation), with a final extension at 72˚C for 300 seconds. Negative controls were included for each amplification and barcoded primer pair, including amplification without template DNA. The presence of amplicons was confirmed by gel electrophoresis on a 2% agarose gel and staining with ethidium bromide. PCR products were quantified

using Quant-iT PicoGreen dsDNA assay. Equimolar amounts (50 ng) of the PCR amplicons were mixed in a single tube. Amplification primers and reaction buffers were removed using the AMPure Kit (Agencourt, Beverly, MA) and purified amplicon mixtures sequenced by Roche/454 FLX pyrosequencing using 454 Life Sciences primer A by the Institute for Genome Sciences, University of Maryland School of Medicine, using protocols recommended by the manufacturer. All 16S rRNA gene amplicons were sequenced as part of the same pool in the same sequencing reaction.

2.3.7 Sequence processing and estimation of bacterial diversity

16S rRNA gene sequences were processed using a combination of QIIME (Caporaso *et al*., 2010) and Mothur (Schloss *et al*., 2009). Pyrosequences were binned using sample-specific barcodes and trimmed by removal of barcodes and primer sequences using default parameters in QIIME (http://qiime.org/scripts/split_libraries.html). Chimeras were removed with UCHIME (Edgar *et al*., 2011). Pyrosequences were rigorously filtered as described by Huse *et al*. (2010), using Mothur. Sequences were removed from the analysis if they were <400 bp, and contained ambiguous characters. To ensure that the V1-V3 region was targeted, all sequences (including isolates and clones) were cut to 500 bp for the analysis. Following quality filtration, sequences were clustered at 3% with the average neighbor-joining method. The Yue and Clayton theta similarity coefficient was used to compare bacterial community structure (Yue and Clayton, 2005, Schloss *et al*., 2009). Phylogenetic analyses were performed with the ARB software package (Ludwig *et al*., 2004) and phylogenetic trees were constructed using the neighbor-

joining algorithm (Saitou and Nei, 1987). Bootstrap values were generated using PHYLIP with 1000-replicate data sets.

2.3.8 Epifluorescence imaging and MALDI/MS analysis

MALDI-MS imaging of a longitudinal 14-µm thick section of an individual *E. rufescens* was performed as described by Simmons *et al.* (2008). The epifluorescence image obtained at 590 nm was overlaid by the MALDI image at m/z 1500 to obtain differential localization of the known compound KF.

2.4 Results

2.4.1 Bacterial community analysis and methods

In addition to characterizing the bacteria I was interested in culturing representative bacteria that may be important to the sacoglossan host. Bacterial communities were characterized with four different methods, which provided consistent results: 1) DGGE, 2) short fragment 16S rRNA gene amplicon pyrosequencing, 3) cloning by partial-length 16S rRNA gene amplicon Sanger sequencing, and 4) cultivation and characterization of isolates by partial-length 16S rRNA gene Sanger sequencing. All 16S rRNA gene assignments were made at the species level.

The bacterial community structure associated with three individuals of *E. rufescens* (ER1-3), two samples of its mucus (M1-2), and surrounding water samples (W1-3) were initially compared using DGGE coupled with BioNumerics clustering analysis (Fig. 2.1A, B). Although DGGE has limited sensitivity for detection of rare community members, cluster analysis showed that bacterial communities in replicate

samples of *E. rufescens*, mucus and water were consistent while the communities in the *E. rufescens* and mucus were more similar to each other than to those from water samples. DGGE analysis indicates major differences in the bacterial communities associated with *E. rufescens* compared with surrounding seawater.

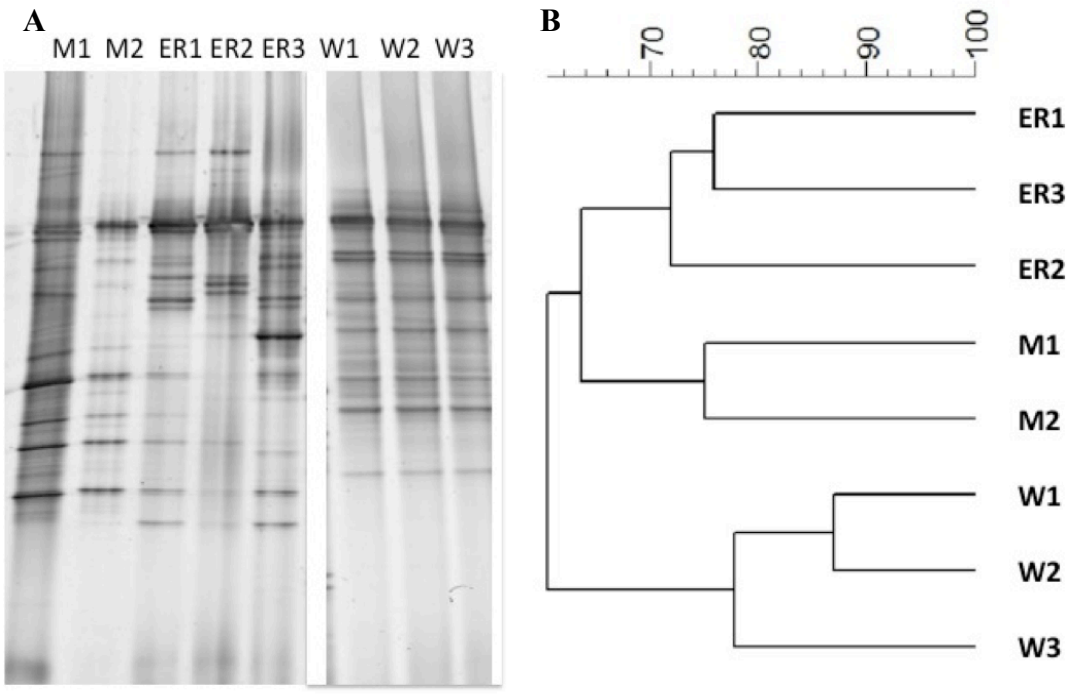


Figure 2.1. DGGE fingerprints of the bacterial communities associated with samples in study. *E. rufescens*, secreted mucus from *E. rufescens*, and surrounding seawater samples (A). UPGMA dendrogram showing clustering of normalized DGGE. The scale bar represents % similarity (B).

A total of 29,063 16S rRNA gene sequences were then analyzed from three individual *E. rufescens* and two mucus samples (Table 2.1). Datasets from these three individuals of *E. rufescens* contained 10,405 (ER1), 9,108 (ER2), and 1,230 (ER3) 16S rRNA gene pyrosequences and were together assigned to 1,225 OTUs after the removal of 6,110 chloroplast-derived sequences (Table 2.1 and Table 2.2). Datasets from the two mucus samples from *E. rufescens* contained 6,533 (M1), and 1,539 (M2) 16S rRNA gene pyrosequences and were together assigned to 1,873 OTUs after the removal of 5 chloroplast-derived sequences (Table 2.1 and Table 2.2). Rarefaction curves did not reach a plateau for any sample (Fig. 2.2), indicating further sampling is needed to determine the full diversity of the bacterial communities associated with *E. rufescens* and its secreted mucus at the species level.

For the characterization of samples by partial-length 16S rRNA gene amplification, 96 clones containing 16S rRNA genes were analyzed from *E. rufescens*, of which only 16 were not chloroplast-derived and assigned to 6 OTUs while 87 clones were analyzed from the secreted mucus, which did not contain any chloroplast-derived reads and were assigned to 15 OTUs (Table 2.1).

Cultivable isolates from *E. rufescens* and mucus samples were randomly picked and identified by partial-length sequencing. These isolates were assigned to 6 OTUs for *E. rufescens* and 7 OTUs for secreted mucus (Table 2.1).

Table 2.1. Number of 16S rRNA gene sequences analyzed and number of OTUs observed from *E. rufescens* and its mucus.

Source of genetic material	Total no. of sequences	No. of chloroplast-derived sequences removed	No. of OTUs
Elysia rufescens			
Isolates	35	----	6
Clones	96	80	6
Pyrosequences	20,743	6,110	1,225
Mucus			
Isolates	30	----	7
Clones	87	----	15
Pyrosequences	8,072	5	1,873

*Distance of 0.03.

Table 2.2. Number of 16S rRNA gene pryosequences analyzed with chloroplast-derived sequences, number of OTUs observed, and richness estimator for *E. rufescens* and its mucus.

	Elysia rufescens			*Mucus*	
	ER1	ER2	ER3	M1	M2
No. Sequences w/o chloroplast	4727	9108	803	6528	1539
No. OTUs	485	650	168	1551	406
Good's Coverage Estimator	91%	93%	83%	79%	77%

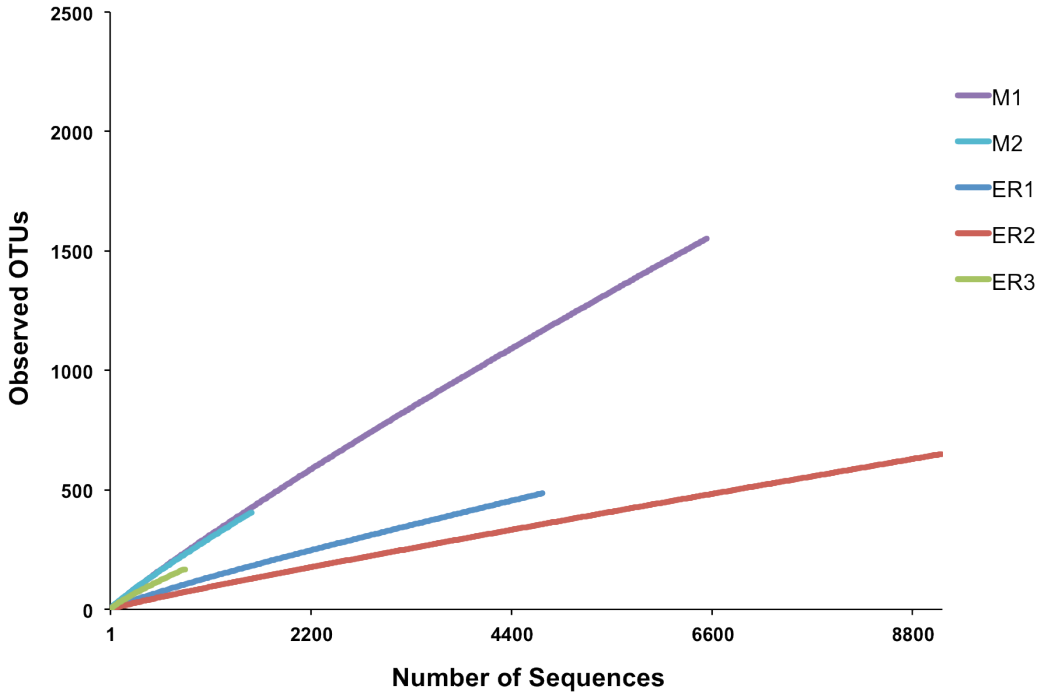


Figure 2.2. Rarefaction curves for *E. rufescens* and its mucus. Shown are rarefaction curves for the 16S rRNA gene sequences from *E. rufescens* (ER1, ER2, ER3) and secreted mucus from *E. rufescens* (M1 and M2).

2.4.2 Abundant bacterial groups

16S rRNA gene sequences affiliated with the phylum *Tenericutes* were abundant in the bacterial communities of both *E. rufescens* and its mucus (90% and 25% of the sequences, respectively) (Figure 2.3A). The three individuals of *E. rufescens* varied greatly with respect to the percentage of sequences assigned to different bacterial phyla but *Tenericutes* was the most abundant phylum associated with all three *E. rufescens* individuals, including the clones (Figure 2.3B). *Gammaproteobacteria* were the most abundant bacterial group associated with the

mucus pyrosequences (33%) and clones (38%) (Figure 2.1A and 2.B). *Alphaproteobacteria* (24%) and *Spirochaetes* (11%) were also abundant in the mucus of *E. rufescens*. The secreted mucus samples were similar with respect to the percentage of sequences assigned to bacterial taxa and *Gammaproteobacteria* was the major bacterial group in both replicates. All isolates cultured from secreted mucus were assigned to *Gammaproteobacteria* and represented over 60% of the isolates cultured from *E. rufescens* (Figure. 2.3B). *Bacteroidetes* were present in the cultured bacteria from *E. rufescens* and in the pyrosequences of the secreted mucus (Figure 2.3B).

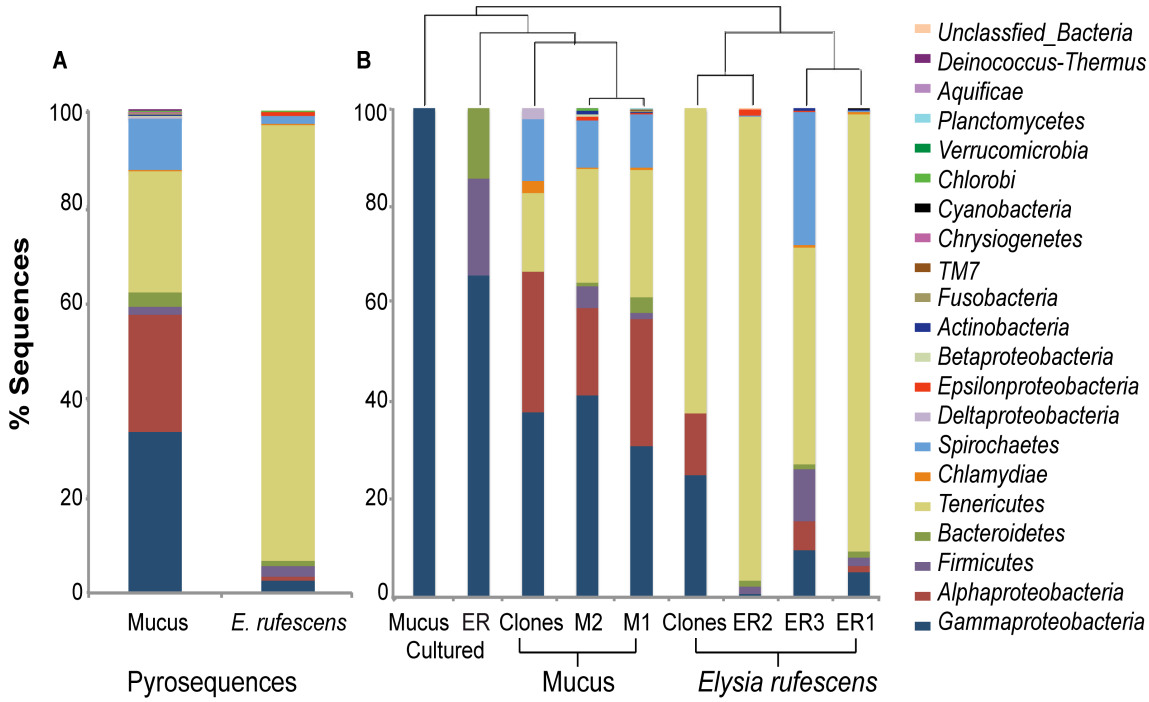


Figure 2.3. Percentage of total sequences representing various bacterial phyla. Combined pyrosequences from secreted mucus from *E. rufescens* and total *E. rufescens* (A) and from all samples (B). OTUs were defined by 3% sequence difference from the nearest neighbor. Groups are shown in the order in which they are listed.

2.4.3 Classification of abundant bacterial groups *Tenericutes* and *Gammaproteobacteria*

All of the *Tenericutes*-derived sequences were assigned to one genus, *Mycoplasma*. At least 50% or more of the *Gammaproteobacteria*-derived sequences belong to the family *Vibrionaceae* for all samples and methodologies except the pyrosequences of *E. rufescens* (Figure 2.4A). However *Vibrionaceae* was still

abundant in the pyrosequences of *E. rufescens*, comprising 22% of the *Gammaproteobacteria*-derived sequences (Figure 2.4A). *Piscirickettsiaceae* and *Oceanospirillaceae* were also abundant in the pyrosequences of *E. rufescens* (34% and 23%, respectively) as well as in the clones but were rare in the mucus pyrosequences and clones. At the genus level, over 65% of the *Gammaproteobacteria*-derived sequences belong to *Vibrio* spp. for all samples and methods (Figure 2.4B).

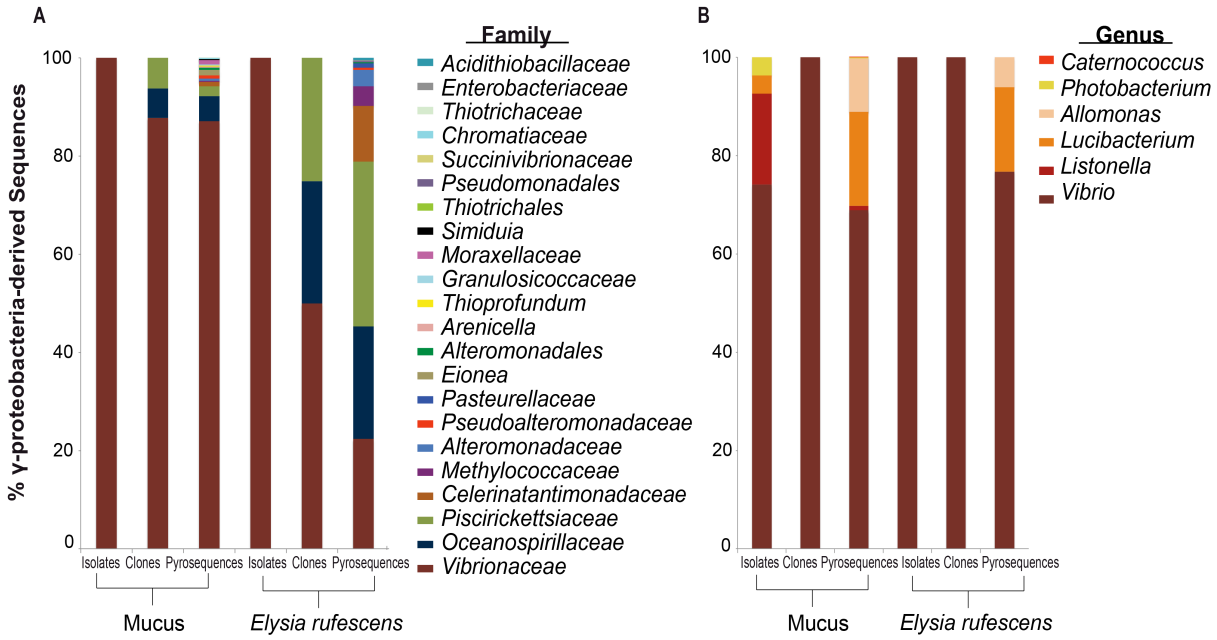


Figure 2.4. Percentage of total *Gammaproteobacteria*-derived sequences from secreted mucus from *E. rufescens* and total *E. rufescens*. *Gammaproteobacteria*-derived sequences classified at the family (A) and genus (B) level. OTUs were defined by 3% sequence difference from the nearest neighbor. Groups are shown in the order in which they are listed.

2.4.4 Classification of *Bacteroidetes*

Flavobacteria were the only cultured representative of the *Bacteroidetes*-derived sequences and were also found in the pyrosequences of the secreted mucus and whole *E. rufescens* (Figure 2.5A). *Flavobacteria* represented 20% and 60% of the *Bacteroidetes*-derived sequences in the two mucus samples but represented less than 1% of the *Bacteroidetes*-derived sequences from *E. rufescens*. At the genus level, *Tenacibaculum* dominated one mucus sample and the cultured bacteria from *E. rufescens* (Figure 2.5).

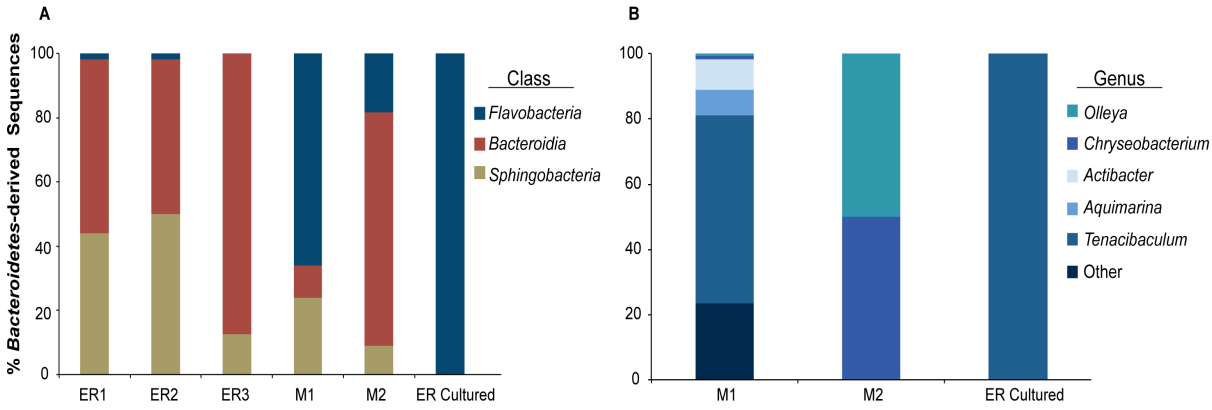


Figure 2.5. Percentage of total *Bacteroidetes*-derived sequences from total *E. rufescens* and secreted mucus from *E. rufescens*. *Bacteroidetes*-derived sequences classified at the class (A) and genus (B) level. Samples that contained less than 1% *Flavobacteria* are not shown at the genus level. OTUs were defined by 3% sequence difference from the nearest neighbor. Groups are shown in the order in which they are listed.

2.4.5. Shared OTUs

Gammaproteobacteria (*Vibrio* spp.) were the only OTUs shared across all samples and method types. There were a total of two OTUs shared by the isolates and pyrosequences of *E. rufescens* and one OTU shared between the isolates and clones of *E. rufescens*, all classified as *Vibrio* spp. The clones and pyrosequences of *E. rufescens* shared five OTUs, belonging to *Gammaproteobacteria* (4 OTUs) and *Mycoplasma* (1 OTU) (Figure 2.6A and Table 2.3). *E. rufescens* samples shared one OTU that was found in the cultured isolates, clones, and pyrosequences and was identified as ER-OTU-1, *Vibrio* sp. (Figure 2.7). This OTU was 100% identical to *Vibrio* sp. UST061013-043 (GenBank Acc. No.: EF587982).

The secreted mucus isolates and pyrosequences shared seven OTUs and the isolates and clones shared two OTUs, all were classified as *Gammaproteobacteria* and seven of the nine OTUs were *Vibrio* spp. (Figure 2.6B and Table 2.3). The clones and pyrosequences of the mucus shared eleven OTUs, *Gammaproteobacteria* (5 OTUs), *Alphaproteobacteria* (2 OTUs), *Spirochaetes* (2 OTUs), *Chlamydia* (1 OTU), and *Tenericutes* (1 OTU). Secreted mucus samples shared two OTUs found in the cultured isolates, clones and pyrosequences, which were identified as ER-OTU-1 and ER-OTU-2 (Figure 2.7). The closest relative in Genbank of ER-OTU-2 is an uncultured bacterium clone PMFP60 (GenBank Acc. No.: AB739857) isolated from the mollusk, *Pinctada margaritifera* and *Vibrio shilonii* HE9 (GenBank Acc. No.: FN554598) isolated from the gut of mollusk, *Haliotis diversicolor*. Both of the closest relatives share 99% identity to ER-OTU-2.

The pyrosequences of *E. rufescens* and its mucus shared 92 OTUs, including 27 OTUs assigned to *Mycoplasma* and 20 OTUs assigned to *Gammaproteobacteria* (Figure 2.6C and Table 2.3). These 92 shared OTUs contain over 19,000 sequences and represent nearly 84% of the total pyrosequences associated with *E. rufescens* and its mucus.

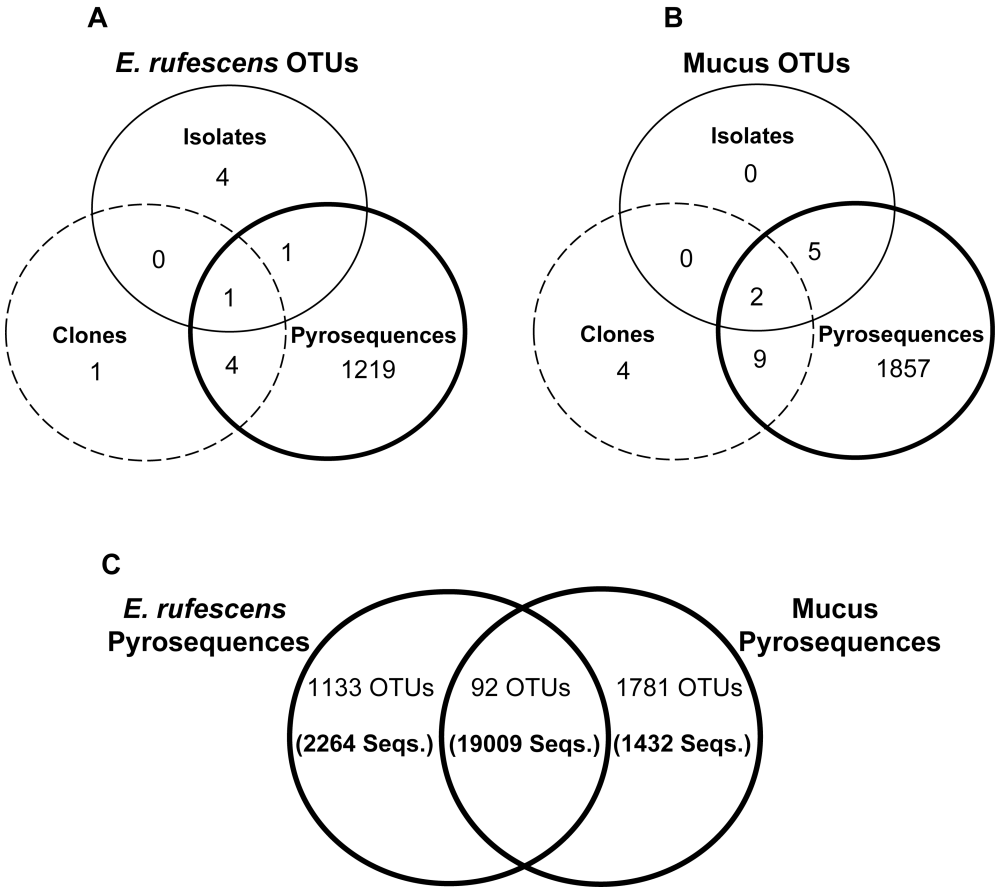


Figure 2.6. Shared OTUs. OTUs that are shared between total *E. rufescens* (A), mucus secreted from *E. rufescens* (B) and pyrosequences from *E. rufescens* and its mucus (C). The number of shared pyrosequences is given in parenthesis. OTUs were defined by 3% sequence difference from the nearest neighbor.

Table 2.3 Bacterial Classification of shared OTUs

	E. rufescens		
	Isolates & Clones	Isolates & Pyrosequences	Clones & Pyrosequences
Shared OTUs	1	2	5
Classification of OTUs	γ-*Proteobacteria* *Vibrio* sp.	γ-*Proteobacteria* *Vibrio* sp.	4 γ-*Proteobacteria* 2 *Vibrio* sp. *Neptunomonas* sp. *Methylophaga* sp. *Tenericutes* *Mycoplasma* sp.
	Mucus		
	Isolates & Clones	Isolates & Pyrosequences	Clones & Pyrosequences
Shared OTUs	2	7	11
Classification of OTUs	2 γ-*Proteobacteria* *Vibrio* sp.	γ-*Proteobacteria* 5 *Vibrio* sp. *Listonella* sp. *Photobacterium* sp.	5 γ-*Proteobacteria* 3 *Vibrio* sp. *Neptunomonas* sp. *Methylophaga* sp. 2 α-*Proteobacteria* Kiloniella sp. 2 *Spirochaetes* Exilispira sp. *Chlamydia* *Simkania* sp. *Tenericutes* *Mycoplasma* sp.
	***E. rufescens* and Mucus Pyrosequences**		
Shared OTUs	92		
Classification of OTUs	27 *Tenericutes* *Mycoplasma* sp. *Epsilonproteobacteria* *Helicobacter* sp. *Actinobacteria* *Rothia* sp. *Chlamydiae* *Simkania* sp. *Betaproteobacteria* *Neisseria* sp. *Fusobacteria* *Streptobacillus* sp. *Chrysiogenetes* *Chrysiogenes* sp.	20 γ-*Proteobacteria* 8 *Vibrio* sp. 5 *Neptunomonas* sp. 2 *Methylophaga* sp. *Teredinibacter* sp. *2 Celerinatantimonas* sp. *Psychrosphaera* sp. *Cycloclasticus* sp. 13 *Firmicutes* *6 Streptococcus* sp. 2 *Gemella* sp. *2 Bulleidia* sp. *Oribacterium* *Oscillibacter* sp. *Veillonella* sp.	*13 α-Proteobacteria* 8 *Kiloniella* sp. 4 *Pseudorhodobacter* sp. *Hellea* sp. *4 Bacteroidetes* *3 Prevotella* sp. *Anaerorhabdus* sp. 5 *Spirochaetes* 4 *Exillispira* sp. *Turneriella* sp.

2.4.6 Phylogeny of shared *Gammaproteobacteria*-derived OTUs

Nine *Gammaproteobacteria*-derived OTUs were shared across multiple methodologies and samples and six of those OTUs were closely related to *Vibrio* spp. (Figure 2.7). The most dominant OTUs of this family include ER-OTU-1 (935 sequences) found in all samples and ER-OTU-2 (523 sequences) found in all samples except the *E. rufescens* clones. These two OTUs represented 58% and 32% of the total sequences assigned to *Vibrio* spp. respectively. ER-OTU- 9, ER-OTU-8, and ER-OTU-11 were exclusively found associated with the mucus samples and two of the three OTUs closest relatives were isolated from the mucus of a coral or from mollusks (Figure 2.7). The closest relative to ER-OTU-13 based on BLAST analysis is *Vibrio marisflavi* and this OTU was found in the clone and pyrosequence analysis of the *E. rufescens* bacterial community as well as by the pyrosequence analysis of the mucus-associated bacterial community. There was one *Gammaproteobacteria*-derived shared OTU found in the family *Oceanospirillaceae*, ER-OTU-15 (49 sequences), and the sequences from this OTU represented 22% of the total sequences in this family. This was a novel shared OTU with 92% identity to an uncultured bacterial clone and 89% identity to the cultured representative, *Neptonomonas japonica*. One *Gammaproteobacteria*-derived shared OTU belonged to the family *Piscirickettsiaceae*, ER-OTU-14 (136 sequences) and the sequences affiliated with this OTU represented 67% of the total sequences in the family. This was also a novel OTU with 95% identity to an uncultured *Methylophaga* sp. clone and 91% identity to the closest cultured relative *Methylophaga sulfidovarans*.

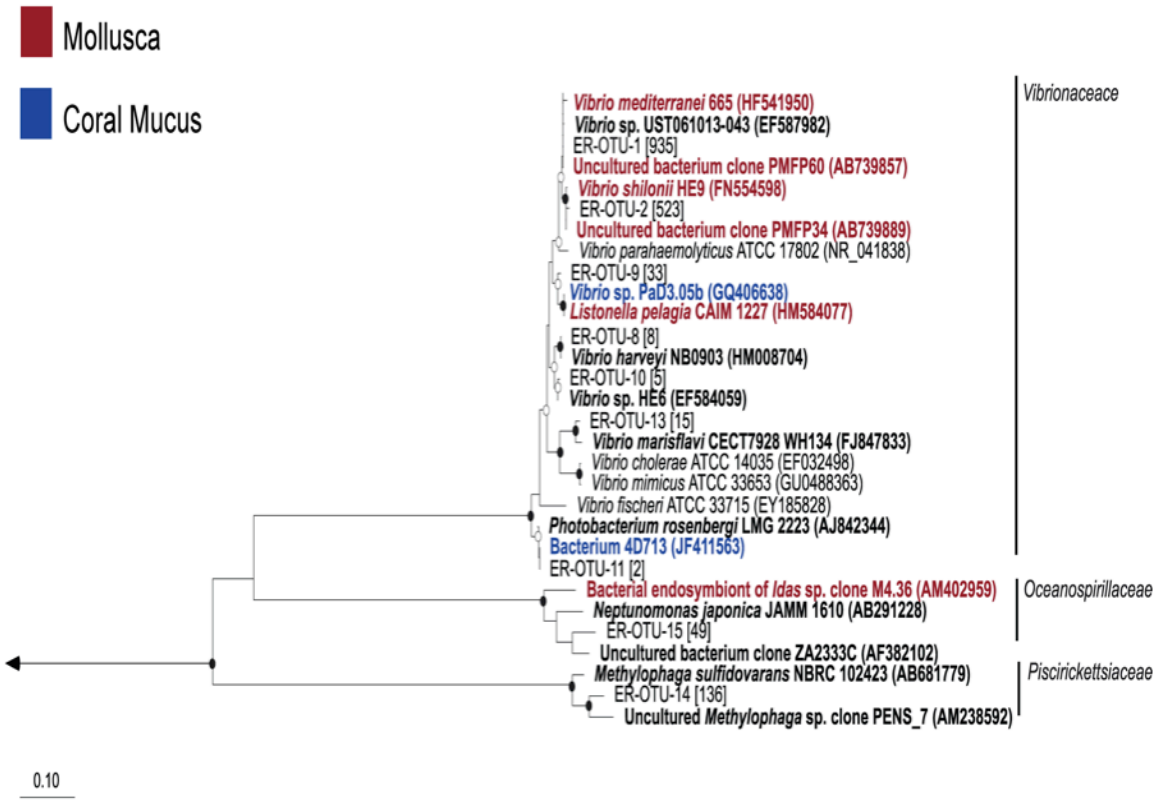


Figure 2.7. Neighbor-joining tree of *Gammaproteobacteria*-derived shared OTUs. The number of sequences in each OTU is listed in brackets and the nearest neighbors in bold. The nearest neighbors highlighted in red were isolated from Mollusks while the nearest neighbors highlighted in blue were isolated from the mucus of a coral. Bootstraps values (neighbor-joining method, 1000 replicates) are indicated by closed circles (values >90%) and open circles (values>65%). The arrow goes to an outgroup, *Streptomyces albolongus* (AB184425). The scale bar represents 10% sequence divergence.

2.4.7 Phylogeny of dominant *Mycoplasma*-derived OTUs

Eleven OTUs dominated (>10 sequences) the abundant bacterial group *Tenericutes* and all OTUs were *Mycoplasma* spp. *Mycoplasma*-derived OTUs clustered between common shared relatives, uncultured *Mycoplasma* sp. clones isolated from *Bryopsis* sp. and uncultured *Mycoplasma* sp. clones isolated from the digestive tract of mollusks (Figure 2.8). The most dominant shared OTUs, ER-OTU-71 (5,298 sequences) and ER-OTU-12 (8,895 sequences), together represented 93% of the total sequences assigned to *Mycoplasma* and were found in both the cloned and pyrosequenced samples of *E. rufescens* and its mucus. ER-OTU-71 was 97% identical to an uncultured *Mycoplasma* sp. clone MX19.9 (GenBank Acc. No.: JF521606) isolated from *Bryopsis* sp., with only 92% coverage while ER-OTU-12 was 90% identical to an uncultured *Mycoplasma* sp. clone U19 (GenBank Acc. No.: AB621843) isolated from the abalone *Haliotis gigantea*. *Mycoplasma*-derived OTUs clustered with uncultured *Mycoplasma* sp. clones and were distantly related to well-known *Mycoplasma* type strains and do not group with the only other *Mycoplasmataceae* genus *Ureaplasma* sp.

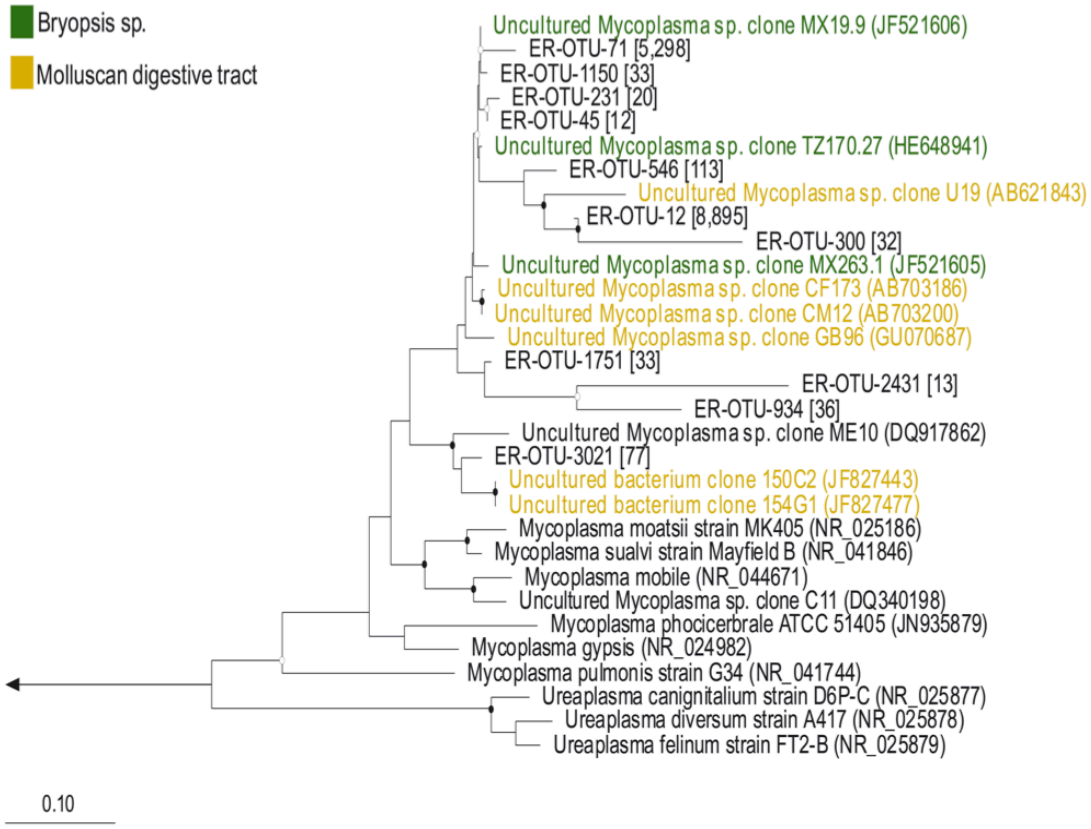


Figure 2.8. Neighbor-joining tree of the dominant *Mycoplasma*-derived OTUs. The number of sequences in each OTUs is listed in brackets and the nearest neighbors are in bold. The nearest neighbors highlighted in green were isolated from *Bryopsis* sp. while the nearest neighbors highlighted in yellow were isolated from the digestive tract of a Mollusk. Bootstraps values (neighbor-joining method, 1000 replicates) are indicated by closed circles (values >90%) and open circles (values>65%). The arrow goes to an outgroup, *Nitrosomonas europaea* (AB070982). The scale bar represents 10% sequence divergence.

2.4.8 Epifluorescence and MALDI-MS imaging

Imaging techniques confirmed the presence of KF in the sea slug, *E. rufescens*. The sea slug and algal diet *Bryopsis* sp. form a close association (Figure 2.9A). Epifluorescence microscopy revealed the autofluorescence of chloroplasts associated with the sacoglossan (Figure 2.9B). Autofluorescence imaging overlaid by MALDI-MS imaging revealed the presence of the KF compound in the outer region of an *E. rufescens* individual (Figure 2.89C). It was not possible to distinguish whether the KF is present in the outer tissue of the *E. rufescens* or in the mucus surrounding the mollusk.

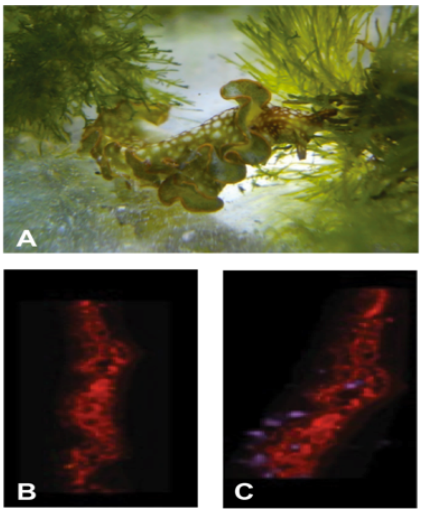


Figure 2.9. Images of *E. rufescens*. *E. rufescens* feeding on algae diet *Bryopsis* sp. in the Institute of Marine and Environmental Technology (IMET) Aquaculture Research Center (ARC) (A). Autofluorescence of *Elysia rufescens* 14 μm longitudinal thin section at 590 nm; (B) Autofluorescence (590 nm) overlaid by MALDI image of $[M + Na]^+$ at m/z 1500 (purple), indicating presence of compound with mass spectrum consistent with kahalalide F (C).

2.5 Discussion

My study provided insights into the bacterial communities associated with *E. rufescens* and its secreted mucus by using a variety of methods that consistently uncovered the presence of the same abundant bacterial groups. Results presented here revealed that although *E. rufescens* and its secreted mucus are hosts to many of the same bacterial groups, the bacterial communities are not identical. Two bacterial groups, *Tenericutes* (*Mycoplasma* spp.) and *Gammaproteobacteria* (mainly *Vibrio* spp.), were the most abundant in the bacterial communities associated with total *E. rufescens*, and secreted mucus from *E. rufescens*, respectively. *Vibrio* spp. were the most abundant cultured bacteria and were also abundant in the pyrosequences. Notably, the phylogenetic analysis revealed that in most cases the closest relatives of the shared OTUs assigned to *Vibrio* spp., were isolated from other mollusks or the mucus layer of invertebrates, whereas the closest relatives of *Mycoplasma*-derived OTUs were uncultured clones obtained from *Bryopsis* sp., the algae diet of *E. rufescens* or the gut of other mollusks that are known to forage on algae.

Bacteria belonging to the genus *Vibrio* are well recognized for their symbiotic relationships, pathogenicity and the production of bioactive secondary metabolites (Ruby, 1996; Faruque *et al*., 1998; Wietz *et al*., 2010; Mansson *et al*., 2011). Opisthobranchs are known to secrete metabolites in their mucus as a defense mechanism (Marín and Ros, 2004). The abundance and concentration of vibrios in the mucus combined with their well-documented metabolic flexibility makes it tempting to speculate that they are candidates to be the producers of the defense

chemical KF found in *E. rufescens*. The MALDI-MS imaging of KF does reveal that the compound is present in the outer region of the *E. rufescens*, conceivably in the mucus. However, it is important to note that we cannot be certain that the KF is in the mucus and there is no direct connection between the presence of KF in the outer region of the mollusk and the dominance of Vibrios in the mucus revealed in this study. Also, no metabolic functions were determined for the Vibrios.

Mycoplasma spp. are a diverse group of bacteria and are known for pathogenicity in a wide range of mammalian hosts (Whithear, 2001). Recent studies reveal that they are members of microbial communities associated with isopods, fish and abalone where they may provide nutritional benefits to the host (Fraune and Zimmer, 2008; Bano *et al*., 2007; Huang *et al*., 2010). In the bacterial community analysis of *E. rufescens* and its mucus, all sequences assigned to *Tenericutes* belong to the genus *Mycoplasma*. However, no *Mycoplasma* was cultured and it is not known if the sequenced *Mycoplasma* comes from the algal diet *Bryopsis* sp. or from the *E. rufescens*. The closest relatives of the dominant OTUs were uncultured *Mycoplasma* spp. clones obtained from *Bryopsis* spp. or the digestive tract of other mollusks, specifically the abalone of the genus *Haliotis*. A study performed on *Haliotis* sp. found that it could feed on a wide range of algae (Naidoo *et al*., 2006). The findings of Erasmus *et al*. (1997) suggest that bacterial members of the digestive tract in *H. midae* possess polysaccharide-degrading ability to assist in breaking down algal diets. More specifically, the closest relative of the uncultured *Mycoplasma* sp. clone obtained from *Bryopsis* sp. in a study preformed on the Mexican coast was the uncultured *Mycoplasma* sp. clone GB69 isolated from *H. diversicolor* (Hollants *et al*.,

2011a) and this is also one of the closest relatives of the dominant *Mycoplasma*-derived OTUs in this study. The presence of *Mycoplasma* sp. in the digestive bacterial community of *H. diversicolor* has also been hypothesized to be algal-food related (Huang et al., 2010; Hollants *et al*., 2011a). It appears that the presence of *Mycoplasma* may be algae specific and might result from algal grazing. Consistent with previous studies, *Mycoplasma* could be a symbiont acquired through the feeding of algal diets; however, this study did not examine any metabolic functions of the *Mycoplasma*.

Both *Vibrio* spp. and *Mycoplasma* spp. are recognized pathogens of marine organisms. A pathogen is traditionally defined as a microbe that causes disease (Casadevall and Pirofski; 2001). Recent microbial community analysis of the most commonly studied host, humans, have revealed that pathogenicity varies in different people, during different times of development and in association with environmental factors (Casadevall and Pirofski, 2000; Casadevall and Pirofski, 2014). For instance, few strains of *E. coli* can cause diarrhea and vomiting, and in one out of three people, *Staphylococcus aureus* simply inhabits nasal cavities without causing harm (Casadevall and Pirofski, 2014; Young *et al*., 2014). Therefore, simply identifying bacteria based on the 16S rRNA gene that are known to be pathogens is not enough information to confer pathogencity. Research in humans demonstrates there are several factors and interactions that shape disease and these factors are attributed to both the host and the associated bacteria (Méthot and Alizon, 2014). We do not yet know if *Vibrio* spp. or *Mycoplasma* spp. are pathogens of *E. rufescens* and/or it algal diet *Bryopsis* sp. It may be of more interest to study the specific interactions between

the hosts and these associated bacterial groups as opposed to if these bacteria are pathogens or not. As demonstrated with the human microbiome, I hypothesize the relationship between *E. rufescens* and its associated bacteria will vary and it is possible that some groups of bacteria may only be pathogenic under specific circumstances.

Flavobacteria are a diverse class within the phylum *Bacteroidetes* and were found in the pyrosequences of the secreted mucus and whole *E. rufescens*, and in the cultured bacteria associated with *E. rufescens*. Species from this class often inhabit seaweed surfaces where they have been shown to play a role in antimicrobial production (Penesyan et al., 2009; Wiese et al., 2009). *Flavobacteria* have been found associated with *Bryopsis* sp. collected along the Mexican coast (Hollants *et al*., 2011a). A recent study analyzed 146 *Bryopsis* samples, covering 23 different species, and 92 additional samples of *Bryopsidales* and indicated host-specificity of *Flavobacteria* endosymbionts that are restricted to *Bryopsis* sp. that are located in warm-temperate and tropical environments (Hollants *et al*., 2013). The presence of *Flavobacteria* may also be algae specific and acquired through algal grazing.

Because the whole samples of *E. rufescens* that were processed for community analysis also contained mucus, higher richness was expected in these whole *E. rufescens* samples than in secreted mucus alone. However, higher bacterial richness was found in secreted mucus samples than in total *E. rufescens*. One possibility for less bacterial richness in whole *E. rufescens* is the uneven distribution of bacterial groups sampled in the community. Almost all of the represented

sequences (93%) were chloroplast-derived or belonged to one single genus, *Mycoplasma* spp., whereas the mucus samples contained more rare phylotypes. This large proportion assigned to only two groups skews the overall bacterial richness and most likely is a result of the intimate association between *E. rufescens* and its diet, *Bryopsis* sp. This symbiotic association enables *E. rufescens* to sequester intact plastids (Rumpho *et al*., 2001), hence the abundance of chloroplast-derived sequences. The alga-sacoglossan relationship may also allow for the exchange of specific bacterial groups such as *Mycoplasma* sp., which as previously noted may result from algal grazing.

Recently, the first insights were provided into the characterization of the bacterial communities from two different *Bryopsis* spp. from the Pacific Mexican coast (Hollants *et al*., 2011b). 16S rRNA gene analysis revealed the presence of *Mycoplasma* sp. clones detected in several *Bryopsis* samples collected hundreds of kilometers apart. A follow-up study by the same group revealed that *Bryopsis* harbors rather stable endophytic bacteria, which showed little variability after one-year cultivation of the algal samples (Hollants *et al*., 2011). Moreover the same uncultured *Mycoplasma* sp. clone remained present over the entire algal cultivation period.

There were a total of 20,743 pyrosequences assessed from *E. rufescens.* Nearly 30% were chloroplast-derived (6,110 sequences) and were removed from the analysis. Of the remaining sequences, 90% were assigned to *Mycoplasma* sp. (13,207 sequences). There were only five chloroplast-derived sequences identified and removed from the secreted mucus samples. *Mycoplasma* spp. were present in both

the whole *E. rufescens* and secreted mucus pyrosequences. However, 87% of the total *Mycoplasma* sequences were from *E. rufescens* samples. The closest known relatives of the most dominant OTUs, ER-OTU-71 (5,298 sequences) and ER-OTU-12 (8,895 sequences) were the same uncultured *Mycoplasma* spp. clones identified previously from *Bryopsis* spp. off the Mexican coast (71). Those OTUs represented 93% of total sequences assigned to the genus *Mycoplasma*. Eliminating the uneven distribution of bacterial groups (Mycoplasma) sampled by further sequencing efforts, would allow for greater bacterial richness observed in total *E. rufescens* as opposed to its secreted mucus. However, the 92 OTUs shared between the pyrosequences of *E. rufescens* and its secreted mucus captured the dominant OTUs of both communities and represent nearly 84% of the total pyrosequences. This demonstrates that although secreted mucus appears to have higher bacterial richness, both *E. rufescens* and its mucus share the same dominant bacterial groups.

The mucus samples not only had greater bacterial diversity than total *E. rufescens* but there also appear to be some rare specific *Vibrio* spp. that are only found in both the cultured and pyrosequenced communities associated with secreted mucus samples. There is one classic example of how mucus secretion assists in the recruitment and selection of specific bacteria. In the bacterial symbiosis between the Hawaiian mollusk, *Euprymna scolopes* and the bioluminescent symbiont *Vibrio fischeri,* host-derived mucus secretion is used to concentrate *V. fischeri* near sites of light organ colonization during initiation of the symbiosis (Nyholm *et al*., 2000). A further study revealed that mucus secretion and ciliated fields are used to recruit nonsymbiotic bacteria as well as *V. fischeri* over the first 24 to 48 hours. However,

once *V. fischeri* successfully colonizes, mucus secretion is suppressed, nonspecific bacteria are no longer recruited or present and only specific strains of *V. fischeri* permanently colonize the light organ (Nyholm *et al*., 2002). In the case of *E. rufescens* perhaps the mucus also selects for specific bacteria that provide functional roles as illustrated in the example above. Indeed copious mucus that bathes and protects sacoglossans is well known (Rumpho *et al*., 2000 Trench, 1975; Paul and Van Alstyne, 1988) and has been a challenge for many working with these organisms (Rumpho *et al*., 1994). However, the dynamics of the mucus layer are not yet known and may possess properties that can influence bacterial composition.

To understand the bacterial community associated with *E. rufescens* and its mucus, we coupled traditional cultivation with cloning and pyrosequencing. Our analysis revealed that the most abundant bacteria captured by culturing, *Vibrio* spp., were also abundant in the culture-independent community, demonstrating the potential importance of *Vibrio* spp. as symbionts of this complex community. While 454 data provided the needed insight into *Mycoplasma* associated bacteria, culturing provided a more comprehensive view of the bacterial community associated with *E. rufescens* when coupled with next generation sequencing.

Chapter 3: Cultured bacterial diversity, phylogeny, and chemical potential of symbionts associated with *Elysia rufescens* and its algal diet *Bryopsis* sp.

3.1 Abstract

Culture-independent techniques reveal complex bacterial diversity associated with marine hosts; however bacteria in pure culture can provide important information about the physiological, pathogenic, and chemical diversity that may not be revealed by culture-independent methods. In this study we used standard culturing methods to assess the cultivable bacteria associated with *E. rufescens* and its algal prey *Bryopsis* sp. over three separate years of collection and obtained over 450 bacteria in pure culture. A total of 156 of these bacteria were identified based on 16S rRNA gene sequencing. *Vibrio* spp. and *Flavobacteria* were the dominant bacteria cultured from *E. rufescens* and *Bryopsis* sp., respectively. In addition, *Vibrio* spp. were screened for signaling molecules and the genome of a representative *Vibrio* sp. was sequenced to give insights into the potential roles of this group of bacteria in the sea slug symbiosis. Lastly, a high-throughput overlay assay was developed and used to screen all cultured bacteria for the production of the defense metabolite kahalalide F.

3.2 Introduction

It is now apparent that many of the bacteria found in the environment have never been cultured and this notion is termed the "great plate count anomaly" (Staley and Konopka, 1985). Many of these bacteria are important for biogeochemical cycling, potential sources of bioactive compounds, and may play significant roles in the ecology of marine hosts (Waksman *et al*., 1933; Bull *et al*., 2000; Das *et al*., 2006; Paul *et al*., 2007). Despite the apparent abundance and huge diversity revealed by 16S

rRNA gene metagenomics, there is a lack of these diverse bacteria in culture. More than half of the known distinct bacterial phyla have no cultivable representatives (Hugenholtz *et al*., 2009). The gap between the bacterial groups revealed only through genetic deep-sequencing analysis and those that have been cultured limits our understanding of the ecology and full genetic diversity of these bacteria. A complete understanding of the functional and physiological characterization of bacteria in their natural environment requires cultivation.

Molecular methods provide great insight into the complex interactions between bacteria. However cultivation provides access to genetic and chemical features of each microorganism that may not be revealed by molecular analyses. Several cultured genera from marine invertebrates demonstrate a wealth of bioactivity (Slattery *et al*., 1995; Zheng *et al*., 2005; Muscholl-Siberhorn *et al*., 2008; Devi *et al*., 2010; Phelan *et al*., 2012); illustrating bacteria associated with marine hosts are a rich source of novel metabolites. Polyketide synthase (PKS) genes encode diverse natural products in several bacteria and for the first time were detected in cultured bacteria from the genus *Aquimarina* (*Bacteroidetes*) in marine sponges (Esteves *et al*. 2013). In comparison to the chemical scaffolds that can be produced by synthetic chemistry, the chemical diversity found in natural products from cultured bacteria is much greater. Novel isolates may produce new compounds that can act as chemical scaffolds for a generation of additional chemical diversity (Joint *et al*. 2010).

Aside from the production of chemical compounds, cultured isolates also reveal ecologically relevant characteristics that may not be apparent through 16S rRNA gene metagenomics. *Vibrio* spp. are ubiquitous in the marine environment and

species can share nearly 99% identity based on the 16S rRNA gene but have strikingly different genomes and the species can perform distinctive roles in the environment (Thompson *et al*., 2004; Gomez-Gil *et al*., 2004). Ecologically *Vibrio* spp. are capable of producing several bioactive compounds and are known to form symbiotic relationships with marine hosts (Mansson *et al*., 2011). The production of the quorum sensing (QS) signal molecules N-acyl homoserine lactones (AHLs) that are involved in cell-cell communication are well studied in *Vibrio* spp. and these molecules can control motility, biofilm production, virulence, secondary metabolite production, and symbiosis (Daniels *et al*., 2004; Parsek and Greenberg, 2005; Zan *et al*., 2012; Williams *et al*., 2007; Griffin *et al*., 2004; Wisniewski-Dye and Downie, 2003). Therefore the ability to culture bacteria, assess their potential roles in the natural environment, and characterize their products remains a significant advantage.

16S rRNA gene characterization of the sacoglossan mollusk *E. rufescens* revealed a diverse assemblage of bacteria in which *Vibrio* spp. were a major component of both the culture and culture-independent analysis (Davis *et al*., 2013). However, there are many unanswered questions surrounding the methods by which bacteria are acquired and maintained, the effects of these bacteria on health of the mollusk, and the potential for symbiotic bacteria to produce the secondary metabolite kahalalide F. Addressing these questions requires extensive research and further laboratory cultivation.

In this study, I used standard culturing techniques to assess the cultivable diversity and phylogeny of bacteria associated with *E. rufescens* and its algal diet *Bryopsis* sp. over three separate years of collection. In addition, I sequenced the

genome of a numerically dominant cultured *Vibrio* sp. isolate associated with this sacoglossan-alga assemblage in hopes of finding the biosynthetic gene cluster that is responsible for KF production. I screened *Vibrio* spp. for QS molecules for further insight on the potential roles of these bacteria. Lastly, I developed a high-throughput screening assay to determine if any these cultured bacteria play a role in the production of the secondary metabolite kahalalide F.

3.3 Experimental methods

3.3.1 Sample collection and processing

Whole *E. rufescens*, its secreted mucus, larvae, the algal diet *Bryopsis* sp., and surrounding water was collected by snorkel at Black Point Bay, Honolulu, HI ($21^{o}15^{'}$N, $157^{o}47^{'}$W). The algal diet that was collected underwater is designated "sea *Bryopsis* sp." while the alga collected on the shore of the beach is designated "beach *Bryopsis* sp." Separate *E. rufescens* individuals were dissected and their parapodia and gut were also processed. All of these samples were collected in the Spring of 2010. *E. rufescens* and *Bryopsis* sp. were also collected in the Spring of 2012 and 2014 in the same location. All samples were processed as described in section 2.3.1.

3.3.2 Bacterial cultivation, identification, phylogeny

Elysia rufescens, its secreted mucus, and its algal diet *Bryopsis* sp. were processed for culture-base analysis and these bacteria were identified as described in section 2.3.2. The phylogenetic analysis was performed as described in section 2.3.7

3.3.3 Genomic sequencing and annotation

Cultured *Vibrio* sp. strain ER1A was isolated from the sea slug *E. rufescens* and sequenced at the Institute of Marine and Environmental Technology (IMET) in Baltimore, Maryland using the Nextera XT kit with 250-bp paired-end read sequencing on an Illumina MiSeq. The genome was assembled with CLC Main Workbench Genome Finishing Module version 7.0 (CLC Inc, Aarhus, Denmark). The genome sequence was annotated and compared to other sequenced genomes by using Rapid Annotation using Subsystem Technology (RAST) (rast.nmpdr.org) (Aziz *et al*., 2008; Overbeek *et al*., 2014). The biosynthetic gene cluster potential was assessed using antiSMASH 2.0 (Blin *et al*., 2013).

3.3.4 N-Acyl homoserine lactone (AHL) screening

A sensitive and broad-spectrum AHL-responsive reporter derived from *Agrobacterium tumefaciens* (At) which cannot synthesize AHLs, but responds to the presence AHLs with acyl chains lengths of C6-C14 (Zhu *et al*., 2003) was utilized to assess isolates for production of AHLs. Marine agar 2216 (Becton Dickinson, Sparks, MD) overlay assays with the At-AHL reporter were used to visualize AHL production as described by Mohamed *et al*. (2008). Briefly, in the strain *Agrobacterium tumefaciens*, *tra*R and *tra*I are homologous to the *lux*R and *lux*I regulatory proteins of *Vibrio fischeri* and the AHLs are similar in structure. The *A. tumefaciens* reporter carried a *lac*Z fusion to the *tra*I and produced a blue color in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), in response to AHLs. Therefore a blue diffusible coloration on a marine agar plate was used to determine if isolates were positive for AHLs.

3.3.5 Development of antifungal overlay assay for kahalaldie F (KF) production

A biological assay for KF production was developed to rapidly and efficiently screen large numbers of isolates. All cultured bacteria associated with *E. rufescens* and *Bryopsis* sp. were purified and transferred to 96 well plates for high-throughput screening of bacteria. KF inhibits the growth of the fungus *Candida albicans* (Hamann and Scheuer, 1993). Therefore, an antifungal assay was established and used to screen isolated bacteria for KF production by detection of zones of growth inhibition in *C. albicans* lawns. To determine the sensitivity of the antifungal assay, a stock concentration of 1 mg of pure KF was dissolved in 1 ml of DMSO, therefore x µl of stock equals x µg dosage of KF. Dosage concentrations of 100 µg, 50 µg, 10 µg, 8 µg, 5 µg, 2 µg and 1 µg of KF were added to a 6 mm paper disk, with concentrations of *C. albicans* ranging from $10^0 – 10^6$ total dilution. It was determined that an overnight culture of *C. albicans* at a concentration of 10^3 total dilution would give the best lawn of fungus and would enable detection of as low as 2 µg of KF (Figure 3.1). A total of four media types were used to screen isolated bacteria; marine agar 2216, 1/10 marine agar 2216, marine agar 2216 with incorporated KF-free *Bryopsis* sp., and 1/10 marine agar 2216 with incorporated KF-free *Bryopsis* sp. Lyophilized *Bryopsis* sp. free of KF was provided by Dr. Mark Hamann's laboratory. All experiments were performed at 30^{o}C in a shaking incubator and in an incubator with no shaking. Bacteria were assayed at days 3, 7, and 21 of growth for KF production.

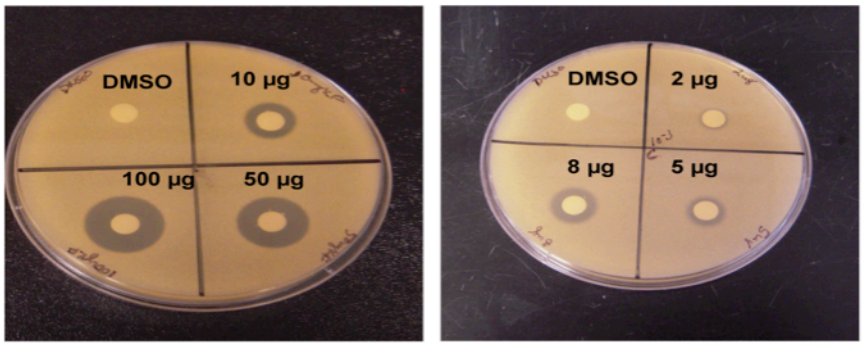


Figure 3.1. Development of antifungal assay. Dosage concentrations of 100 µg, 50 µg, 10 µg, 8 µg, 5 µg, 2 µg and 1 µg of KF were added to a 6 mm paper disk, with *C. albicans* at a concentration of 10^3 total dilution.

3.4 Results

3.4.1 Bacterial classification and phylogeny

Over 140 isolates were identified and *Vibrio* spp. were the dominant cultured bacteria associated with *E. rufescens* and its algal diet *Bryopsis* sp. over three separate years of collection (Figure 3.2). Specifically, *Vibrio* spp. were found to be associated with whole *E. rufescens*, its secreted mucus, sea and beach *Bryopsis* sp., and the seawater samples. Bacteria cultured from *Bryopsis* sp. displayed the most diversity. Isolates from the sea *Bryopsis* were dominated by *Flavobacteria* (*Tenacibaculum*) while isolates from the beach *Bryopsis* were dominated by *Vibrio* spp. *Firmicutes* represented a small proportion of the bacteria cultured from *E. rufescens* and sea *Bryopsis*.

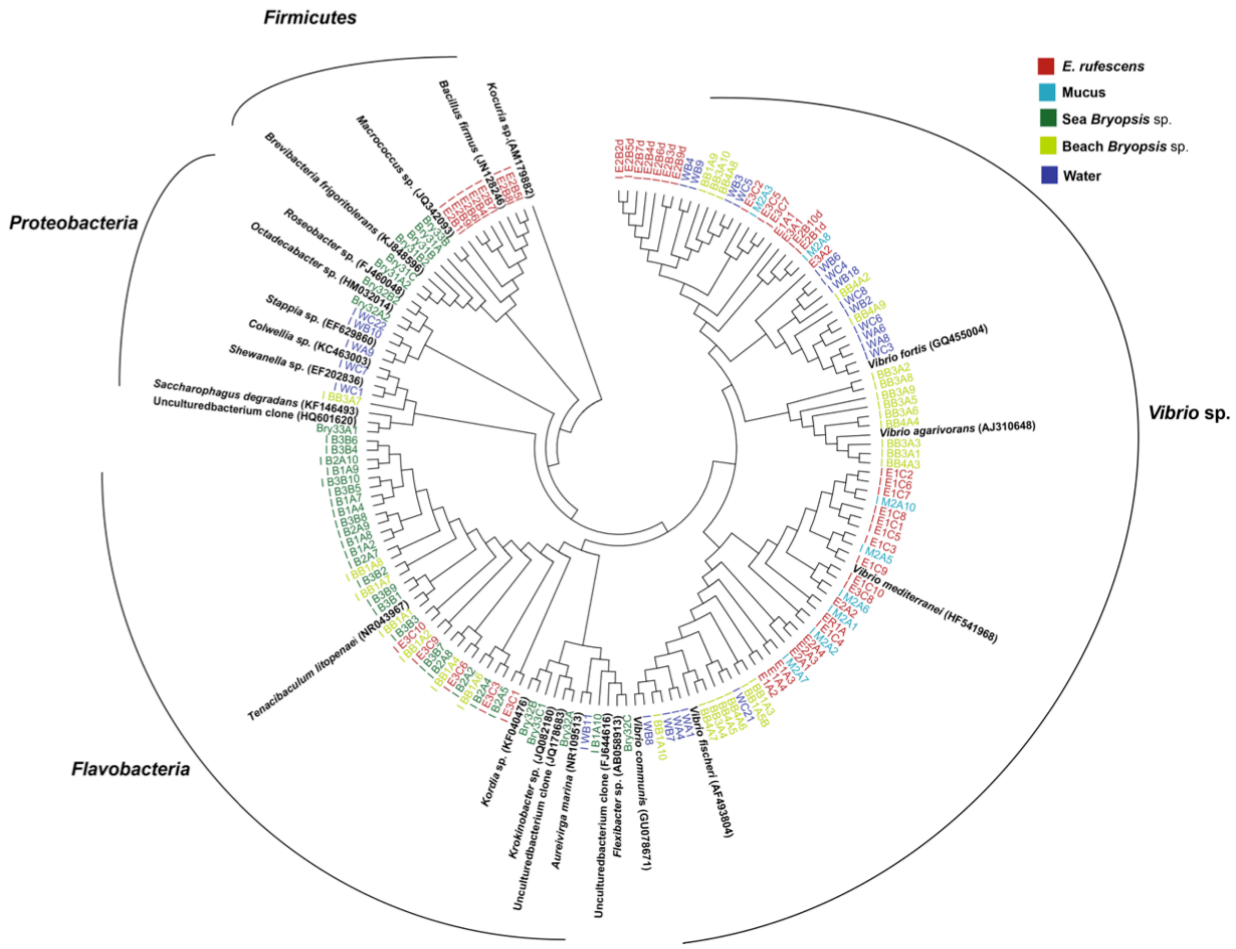


Figure 3.2. Neighbor-joining tree of cultured bacteria associated with *E. rufescens* and *Bryopsis* sp. All isolates are colored and each color represents the isolation source: red, *E. rufescens*, light blue, secreted mucus of *E. rufescens*, dark green, *Bryopsis* sp., from the sea, light green, *Bryopsis* sp. from the beach, and blue, surrounding seawater. The nearest neighbors are bolded.

3.4.2 Assembly and annotation of *Vibrio* sp. strain ER1A

One hundred eight contigs (>1000 bps) with a total size of 6,092,013 bps were assembled from the 3,959,866 raw reads, which resulted in about 140X coverage of *Vibrio* sp. ER1A. The minimum contig length was 1,224 bps and the maximum contig length was 1,593,462 bps. The overall G+C content of *Vibrio* sp. strain ER1A was 44%. A total of 5,202 coding sequences were predicted in the genome of *Vibrio* sp. strain ER1A and were designed to 22 functional categories (Figure 3.3). A major portion of the predicted genes were assigned to carbohydrate metabolism and amino acid synthesis and represented 17.5 % and 12.7% of the total predicted genes. Other major classifications include genes that are involved in cofactors and vitamin production (8%), cell wall and capsule synthesis (5.6%), and membrane transport (6.3%). Interestingly, there were few genes involved in secondary metabolism.

3.4.3 Biosynthetic potential, signaling, and virulence of *Vibrio* sp. strain ER1A

Two biosynthetic gene clusters: a putative nonribosomal peptide synthetase independent siderophore biosynthetic gene cluster and a putative homoserine lactone biosynthetic gene cluster were metabolites revealed in the genome of *Vibrio* sp. strain ER1A through three separate genomic annotation tools, antiSMASH, RAST and BLAST analysis. Genes involved in cell signaling (Lux) were present and displayed 97-100% identity to the Lux genes in *V. shiloni* (Table 3.1). Vibrio polysaccharide (*Vps*) genes involved in biofilm synthesis were present and were 91-100% homologous to the *Vps* genes in *Vibrio* spp. Table). Genes required for siderophore production and iron acquisition (*Iuc* and *Vat*) were also present in the genome and all of these genes displayed 95-100% identity to the corresponding genes in V. *shiloni*.

Well-known genes required for virulence in *Vibrio* spp. such as vibrio pathogenicity island genes and metalloproteases (Zhang *et al*., 2003; Pruzzo *et al*., 2005; Abdallah *et al*., 2013) were absent in the genome.

In addition to the available databases, a separate bioinformatics database was built from the genome of *Vibrio* sp. ER1A to locate specific amino acid sequences required to produce KF. Based on the chemical structure, it has been predicted that KF is produced by a nonribosomal peptide synthetase, an enzyme known to produce peptides that are cyclic and/or branched in structure and are linked amino acids (as in the structure of KF). The adenylation (A) domain is required and selects the cognate amino acid from the pool of available substrates and thus it activates the first amino acid and allows for the loading of amino acids to a growing chain (Marahiel *et al*. 1997). Therefore, the amino acids that make up KF as well as the specific A domains that correspond to the amino acids in KF were searched to find the biosynthetic gene cluster encoding KF in this database. This yielded negative results with no genes being identified that encoded the amino acid sequence found in the KF molecule.

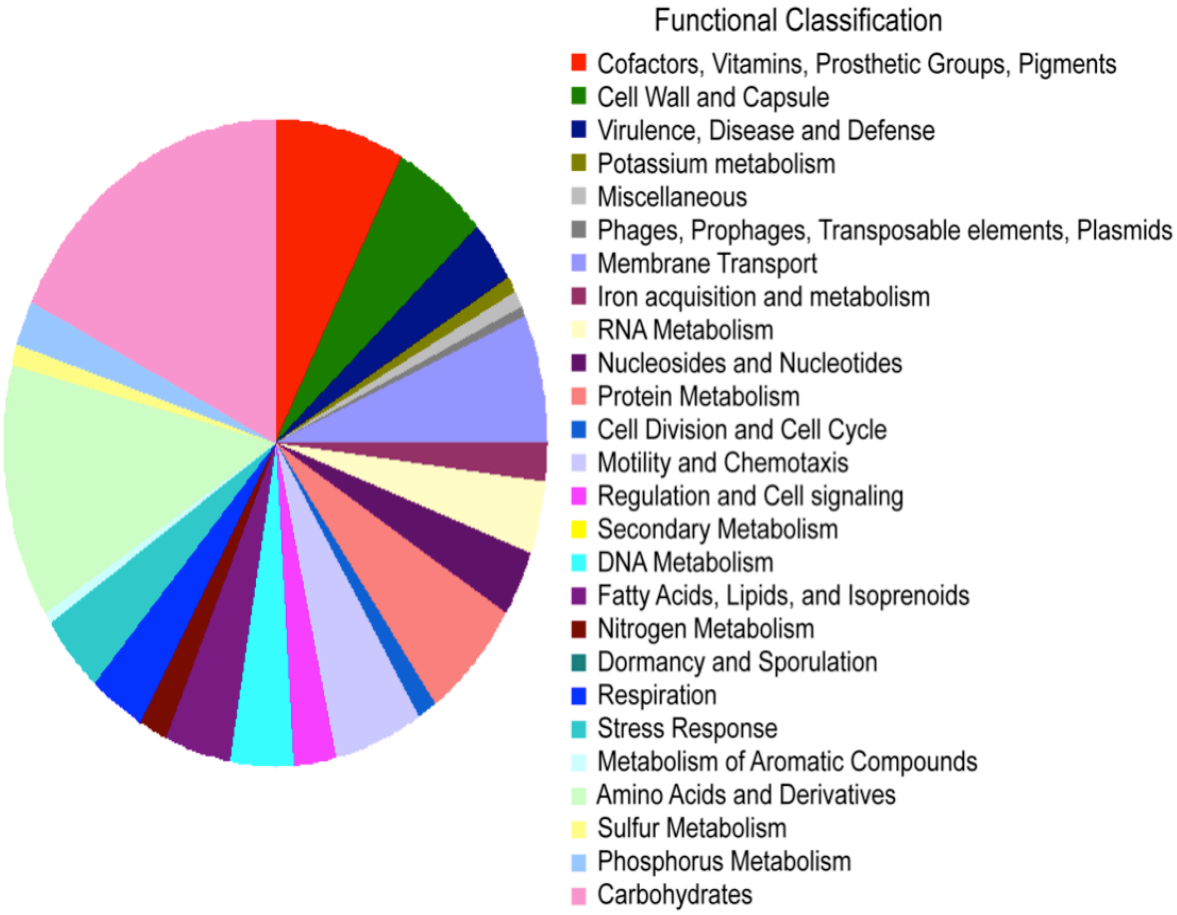


Figure 3.3. Classification of genes in *Vibrio* sp. strain ER1A. Genes in bacterium were classified and grouped into categories. Categories are shown in the order in which they are listed.

Table 3.1. List of highlighted genes found in genome of ER1A and the closest BLAST hit of those genes.

Gene	Function (EC #)	Blast Identity (Isolate) %
Quorum Sensing/ Biofilm formation		
luxS	S-ribosylhomocysteine lyase, autoinducer-2 production protein LuxS in quorum sensing (EC 4.4.1.21)	S-ribosylhomocysteinase (*Vibrio shilonii*) 100%
luxP	Autoinducer 2-binding periplasmic protein LuxP precursor in quorum sensing	LuxPQ_quorum_sensing, ABC transporter substrate-binding protein (*Vibrio shilonii*) 98%
luxQ	Autoinducer 2 sensor kinase/phosphatase LuxQ in quorum sensing (EC 2.7.3.-) (EC 3.1.3.-)	Sensor protein LuxQ (*Vibrio shilonii*) 97%
VpsA	Low molecular weight protein tyrosine phosphatase in *Vibrio polysaccharide* biosynthesis (EC 3.1.3.48)	Protein tyrosine phosphatase (*Vibrio shilonii*) 100%
vpsB	UDP-N-acetylglucosamine 2-epimerase in *Vibrio* polysaccharide biosynthesis (EC 5.1.3.14)	UDP-N-acetylglucosamine 2-epimerase (*Vibrio parahaemolyticus*) 91%
vpsD	Serine acetyltransferase in *Vibrio* polysaccharide biosynthesis (EC 2.3.1.30)	Serine acetyltransferase (*Vibrio shilonii*) 99%
vpsR	Transcriptional regulator VpsR in *Vibrio* polysaccharide biosynthesis	Fis family transcriptional regulator (*Vibrio shilonii*) 99%
hapR	Quorum-sensing regulator of virulence HapR in *Vibrio* polysaccharide biosynthesis	LuxR family transcriptional regulator (*Vibrio shilonii*) 99%
	Transcriptional regulator CdgA in *Vibrio* polysaccharide biosynthesis	GGDEF family protein (*Vibrio shilonii*) 97%
Siderophore/Iron acquisition		
lucA	Citrate:6-N-acetyl-6-N-hydroxy-L-lysine ligase, alpha subunit, aerobactin biosynthesis protein LucA, siderophore synthetase large component, acetyltransferase (EC 6.3.2.27)	Aerobactin siderophore biosynthesis protein LucA (*Vibrio shilonii)* 95%
lucB	N6-hydroxylysine O-acetyltransferase, aerobactin biosynthesis protein LucB, siderophore synthetase small component, acetyltransferase (EC 2.3.1.102)	N(6)-hydroxylysine O-acetyltransferase (*Vibrio shilonii*) 98%
lucD	L-lysine 6-monooxygenase [NADPH], aerobactin biosynthesis protein LucD,siderophore biosynthesis protein, monooxygenase (EC 1.14.13.59)	Lysine 6-monooxygenase (*Vibrio shilonii*) 97%
lutA	Aerobactin siderophore receptor LutA	Aerobactin siderophore receptor iutA (*Vibrio shilonii*) 99%
vatD	Ferric aerobactin ABC transporter, periplasmic substrate binding protein	Peptide ABC transporter substrate-binding protein (*Vibrio shilonii*) 98%
vatC	Ferric aerobactin ABC transporter, ATPase component	Iron ABC transporter substrate-binding protein (*Vibrio shilonii*) 99%
vatB	Ferric aerobactin ABC transporter, permease component	Iron-hydroxamate transporter permease (*Vibrio shilonii*) 99%

3.4.4 AHL screening of Vibrios associated with *E. rufescens* and *Bryopsis* sp.

A total of 73 Vibrios cultured from whole *E. rufescens*, its mucus, algal diet and surrounding seawater was assessed for AHL production (Figure 3.4 A and B). All isolation sources contained five or more *Vibrio* strains capable of AHL production. However Vibrios isolated from entire *E. rufescens* and its secreted mucus yielded the largest proportion of AHL positive bacteria, 74% and 90% respectively (Figure 3.4 B). A total of 32% of the *Vibrio* strains isolated from beach *Bryopsis* sp. were positive for AHL production. The surrounding water yielded the smallest proportion of Vibrios positive for AHL production

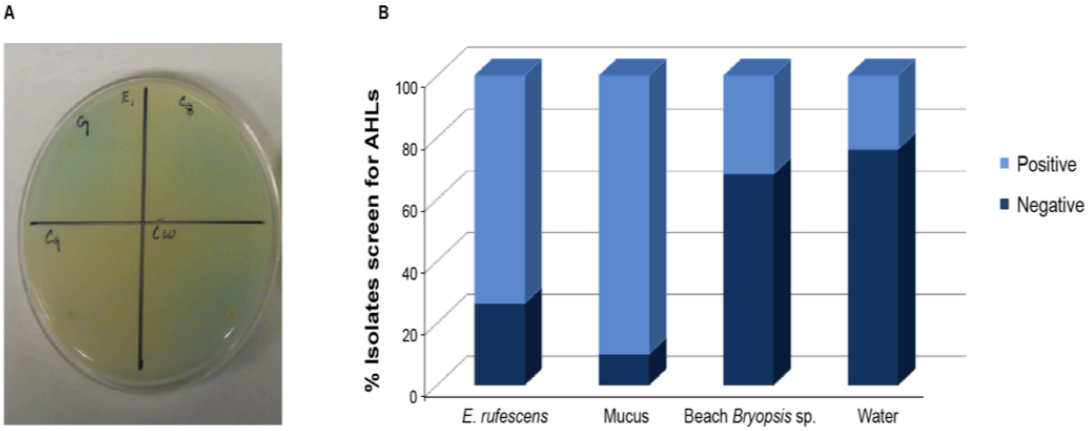


Figure 3.4. Screening of *Vibrio* strains for AHL production. Marine agar bioassay for AHL activity (A) and percentages (B) of AHL-positive and AHL-negative *Vibrio* isolates.

3.4.5 Antifungal screening of cultured bacteria

A total of 460 isolates cultured from whole *E. rufescens*, dissected *E. rufescens*, its algal diet, larvae, and surrounding water were screened for antifungal activity. None were positive for antifungal activity (Table 3.2), which was taken to indicate that these strains were all negative for KF production at the detection limit of the assay. Many bacteria were capable of degrading components of the agar, producing zones of clearing around colonies (Figure 3.5). Bacteria that produced zones in agar were also screened separately to check for false positive results and all were negative for antifungal activity.

Table 3.2. Number of isolates purified and screened for KF production.

Isolation Source	No. cultured bacteria	No. bacteria screened for anti-fungal activity	No. positive for anti-fungal activity
E. rufescens (Whole slug)	90	90	0
E. rufescens (Secreted mucus)	20	10	0
E. rufescens (Larvae)	25	25	0
E. rufescens (Gut)	26	26	0
E. rufescens (Parapodia)	43	43	0
Bryopsis sp. (Sea and Beach)	266	239	0
Surrounding water	27	27	0
		Total = 460 isolates screened	

3.5 Discussion

This study provided insights into the cultured bacteria associated with the chemically defended sacoglossan mollusk *E. rufescens* and its algal food *Bryopsis* sp. Results revealed that *Vibrio* spp. and *Flavobacteria*, specifically *Tenacibaculum* sp. were numerically dominant in the cultivable assemblage. Most of the bacteria cultured from *E. rufescens* were *Vibrio* spp. while the cultivable assemblage of *Bryopsis* sp. was more diverse with respect to phyla. Two distinct bacterial groups were recovered in the *Bryopsis* sp. with *Flavobacteria* comprising the dominant cultured group associated with alga samples from the sea and *Vibrio* spp. dominating the samples on the beach. This reveals the ability for environmental conditions to affect the composition of the cultivable bacterial community.

Many of the well-studied marine Vibrios, *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus* are pathogenic to mollusks and can also be responsible for human diseases when those mollusks are consumed (Thompson *et al*., 2004; Pruzzo *et al*., 2005). With the exception of the mutualistic symbiont *V. fischeri* of the Hawaiian bobtail squid, there is little known about symbiotic *Vibrio* spp., that are not parasitic to their marine host. Consequently there is a need to characterize symbiotic Vibrios to understand their ecological importance in relation to host function and health. Results presented here show that not only are *Vibrio* spp. abundant in the cultivable bacteria associated with *E. rufescens* but are also persistent, as these bacteria remained numerically dominant over three separate collection years. This illustrates that this group of bacteria may be important symbionts of the sacoglossan mollusk. Genomic analysis of the symbiont *Vibrio* sp. strain ER1A revealed that the majority

of the genome is dedicated to carbohydrate metabolism and amino acid synthesis. However, the presence of specific types of gene clusters sheds light on the lifestyle within the sacoglossan host. In particular the presence of the *lux*, *vps*, *iuc*, and *vat* genes demonstrate that the coordination of community behaviors through QS, the formation of biofilm, and the acquisition of iron are potential roles of this bacterium to the sea slug (Thompson *et al*., 2004). In addition, the common virulence genes that are found associated with pathogenic Vibrios (Zhang *et al*., 2003; Abdallah *et al*., 2013) were not present in the genome of *Vibrio* sp. strain ER1A, further suggesting that this strain and perhaps other *E. rufescens*-associated Vibros are not pathogens and may be beneficial. The genome of this strain was not completely sequenced therefore the absence of some genes may be due to the incompleteness of the genome. However, multiple lines of evidence, including the apparent health of the *E. rufescens* from which these bacteria were cultured, support a favorable association of *Vibrio* spp. with *E. rufescens*.

Genes required for cell-cell communication were found in the genome of a representative *Vibrio* sp. associated with *E. rufescens*. Screening for the AHL signaling molecules revealed that a high proportion of the cultured Vibrios associated with the mollusk are capable of cell signaling. Although AHLs were found associated with all screened samples, the highest proportion of AHL positive *Vibrio* spp. was found from *E. rufescens*, especially in the mucus layer where 90% of screened bacteria were AHL positive. Most of the *Vibrio* spp. isolated from the surrounding water did not produce AHL molecules. This may suggest a potential role of AHLs in colonization since more signaling chemicals were found associated

with the bacteria isolated from mollusks compared to those from the surrounding water. The roles of AHL signaling molecules in the mucus layer of several corals have been studied and research suggest QS molecules may play a role in structuring the microbial community of both infected corals with black band disease as well as apparently healthy corals (Alagely *et al*., 2011; Golberg *et al*., 2011; Zimmer *et al*., 2011). The dynamics of mucus secretion are important during symbiont colonization of *V. fischeri* of the bobtail squid that is mediated by the production of AHL molecules and serves as a model for communication in the mucus layer (Nyhom et al., 2002). These examples illustrate the roles of AHLs in the mucus layer of various marine invertebrates in which these communication molecules have the potential to control health, pathogenicity, and colonization.

For many marine invertebrates that have been studied, the cultured bacterial community is not well represented in the total bacterial community that is indicated by molecular analysis. In some cases, cultivable bacteria are completely absent from the community revealed by molecular analysis. For example, Montalvo *et al*. (2014) cultured more than 400 bacteria from two sponges of the genus *Xestospongia* and these bacteria were almost entirely absent from the molecular analysis. However, in the case of *E. rufescens*, the most readily cultured bacterial group, *Vibrio* spp., is a well represented subset of the deep-sequencing bacterial community and is specifically abundant in the secreted mucus of *E. rufescens* (Davis *et al*., 2003). Therefore, the phylogenetic diversity and functional roles presented in this study potentially reflect the diversity and roles of these bacteria found in nature. Molecular identification of Vibrios on the basis of multi-locus sequence analysis may serve as

an added tool to fully assess the phylogeny and diversity (Thompson *et al*., 2005) of the cultured *Vibrio* spp. associated with *E. rufescens* and *Bryopsis* sp.

Over 400 isolates from the sacoglossan-alga assemblage were screened for KF production through an antifungal assay in which no bacterium examined was positive for production of antifungal activity within the detection limits of the assay. There are several possible reasons for the negative results. One reason is that none of these cultured bacteria produce the compound KF. Based on the chemical structure of KF it is generally accepted that the compound is produced by a bacterium. However, standard plating techniques recover a very small proportion of the total bacterial assemblage (Kogure et al., 1979; Amann et al., 1995). Therefore a bacterium associated with *E. rufescens* and *Bryopsis* sp. could be producing KF but may not be readily cultivable, for example if it is harbored intracellularly. Another possibility is the production of KF by a bacterium is below the detection limit of the developed antifungal assay. Therefore a bacterium screened could be responsible for the production of KF but would still give a negative result using this assay. Lastly, bacteria often grow in consortia and interact with other bacteria as well as their marine host. In the absence of these complex interactions some bacteria may no longer produce compounds that are produced under natural conditions. It is feasible that one of the cultured bacteria produced KF, but only under specific growth conditions. To increase the chances of KF production, several media were used including media with the incorporation of KF-free *Bryopsis* sp., which still led to negative results. It is important to note that suitable conditions for KF production is not limited to media types and temperatures but also include the presence of specific

bacteria and host material that are present in the natural environment. There may be no need to produce a secondary metabolite in such rich medium without natural interactions that occur between bacteria and their host.

This study expands our knowledge of the cultivable diversity associated with *E. rufescens* and the potential roles of those bacteria, specifically the numerically dominant *Vibrio* spp. It is still unclear how these bacteria are acquired and if a cultured bacterium is responsible for the production of the defense metabolite KF. However, my data revealed the presence of AHL signaling molecules, which are often involved in colonization and metabolite production and are abundant in the mucus layer where the sacoglossan secrete their defense chemicals (Marín and Ros, 2004). Therefore it is plausible that these molecules may play a role in colonization of bacteria and the metabolite production. Further studies are needed to test this hypothesis. The development of a screening assay allows for quick assessment of antimicrobial chemicals other than KF. Genomic annotation of a symbiotic *Vibrio* sp. that has been continuously cultured over several years and is a major component of the bacterial community associated with *E. rufescens* revealed that this bacterium does not possess the typical genes required for pathogenicity in Vibrios. This work adds to the potential functional roles of symbiotic bacteria, specifically *Vibrio* spp. that are not pathogenic to marine hosts.

Chapter 4: Characterization of the bacterial community of the photosynthetic sacoglossan *Elysia crispata* and associated alga in two distinct Caribbean locations

4.1 Abstract

Sacoglossan sea slugs are unique mollusks and the microbiota associated with mollusks remains largely unexplored. Here I use high-throughput sequencing and microscopy to investigate the bacteria associated with a slug that is both photosynthetic and chemically defended, *Elysia crispata,* from two Caribbean locations, the Bahamas and Puerto Rico, as well as an associated algal diet. Results revealed that Caribbean sacoglossans from different locations harbored low bacterial richness profiles (five phyla representing >96% of the total sequences) that were more similar to each other than to the bacterial communities associated with the slugs' respective alga. The most abundant bacterial group associated with sacoglossan individuals was the recently described phylum *Verrucomicrobia* and these sequences shared only 91-94% identity to its closest known relative, illustrating the abundance of a novel, underexplored bacterial group. *Betaproteobacteria*, *Bacteroidetes,* and *Firmicutes* were also common bacteria associated with *E. crispata* and several groups within those phyla were found associated with other sacoglossans in previous studies. This work forms the basis for describing the bacterial community of the sacoglossan *E. crispata* and furthers our understanding of the potential roles bacteria may play in the unusual sacoglossan niche.

4.2 Introduction

Marine invertebrates engage in intricate interactions with microorganisms, forming symbiotic relationships. Bacteria are often major components of the total microbiota of marine hosts and encompass nearly all major branches of bacterial life (Erwin *et al*., 2014). Sponges and tunicates are known to harbor bacterial lineages

that are maintained regardless of location (Montalvo *et al*., 2011, Dishaw *et al*., 2014), illustrating host-specific bacterial communities.

Bacteria can provide carbon substrate to their marine hosts. Invertebrates can utilize carbon molecules by forming relationships with cyanobacteria (photobionts) to capture photosynthetic products as sources of energy (Pardy, 1980; Pardy and Lewin, 1981; McFarland and Muller-Parker, 1993; Erwin and Thacker, 2008). These types of relationships are most common in the phyla *Porifera* and *Cnidaria* presumably due to the simple body plans and large surface area to volume ratio that facilitates the transfer of nutrients (Venn *et al*., 2008). In theses cases, the photobionts act independently, providing carbon as a source of energy to the host and often receives nutrients in return (Rumpho *et al*., 2011). Sacoglossans have developed a rare mechanism to capture photosynthetic products by harboring solely the photosynthetic organelles as opposed to photosynthetic organisms through a process called kleptoplasty. Although this form of endosymbiosis is common in protists (McManus *et al*., 2012; Gast *et al*., 2007), sacoglossans are the only known animals to perform kleptoplasty.

Several sacoglossans perform photosynthesis using stolen plastids sequestered from their algal diets. However, kleptoplasty varies extensively in duration and function amongst sacoglossan slugs, with some slugs immediately digesting chloroplasts, while others maintain functional plastids for months in the absence of their algal diets (Curtis *et al*., 2010; Pelletreau *et al*., 2011). Species that are able to incorporate chloroplasts but do not photosynthesize are considered no-retention (NR) forms (Clark *et al*., 1990) while others can incorporate chloroplast and

photosynthesize for a few weeks or months during algal starvation and these forms are considered short-term (StR) retention and long-term retention (LtR), respectively (Clark *et al*., 1990; Händeler *et al*., 2009; Christa *et al*., 2014). The NR forms are considered non-functional kleptoplasty while the other forms are considered functional kleptoplasty. In addition to sequestering plastids from their algal diets, sacoglossans may also acquire compounds from their diets through a process called kleptochemistry. These secondary metabolites are often accumulated or modified within the slug and secreted in the mucus as defense molecules (Marín and Ros *et al*., 2004).

Despite the innovations in sequencing technology that have advanced our understanding of the roles of microbial symbionts of marine invertebrates, very little is known about the taxonomic and functional bacterial diversity of sacoglossans. Recent 16S rRNA gene analyses of two distinct populations of the photosynthetic slug *Elysia chlorotica* revealed that these slugs maintain different bacterial communities under varied environmental conditions (wild vs. laboratory), however some bacterial groups were shared between populations (Devine *et al*., 2012). *Elysia chlorotica* demonstrates LtR kleptoplasty with no known chemical defense molecules derived from kleptochemistry while *E. rufescens* possesses known chemical defense compounds derived from kleptochemistry but needs a constant supply of its algal diet to survive; to date these are the only two sacoglossans that have been studied for their bacterial composition.

The sacoglossan *E. crispata* is a sacoglossan that is both chemically defended and photosynthetic. *E. crispata* performs LtR kleptoplasty and maintain functional

photosynthesis up to several months after subsequent starvation of its preferred algal food source (Pierce *et al*., 2006). The ability for *E. crispata* to transform chemicals from its algal diet through kleptochemistry is well documented and this sacoglossan is known to produce *Caulerpa*-derived defense toxins (Gavagnin *et al*., 1997; Gavagnin *et al*., 2000). While the photosynthetic ability and chemical composition of *E. crispata* is known, nothing is known about the bacteria associated with this sacoglossan or the potential roles of bacteria in the unusual sacoglossan lifestyle. In many cases the symbionts may be the true producers of chemical compounds found in invertebrates (Schmidt *et al*., 2005; Piel *et al*., 2004; Davidson *et al*., 2001) and these compounds may be important in bacterial recruitment (Neal *et al*., 2012).

In this study I used extensive 16S rRNA gene sequencing to examine the bacterial communities associated with sacoglossan *E. crispata* collected in two different Caribbean locations, Bahamas and Puerto Rico. A few *E. crispata* individuals from Puerto Rico were found associated with an alga and the bacterial communities associated with this alga were also characterized. The mitochondrial cytochrome oxidase subunit I (COI) genes of the sea slugs were sequenced to identify and investigate the phylogeny of the sacoglossan species. By examining the communities of the sea slug and an algal diet, I investigated what bacteria are present in both the slug and one of the algae on which *E. crispata* feeds. Bacterial groups are conserved by the sea slugs from different geographic locations are likely to be important symbionts that warrant further investigation. Pyrosequencing of *E. rufescens* revealed an abundance of *Mycoplasma*-derived OTUs that were phylogenetically similar to uncultured *Mycoplasma* clones obtained from *Bryopsis* sp.

of Mexico, the algal diet of *E. rufescens* (Davis *et al*., 2013), indicating the possibility of bacteria being obtained from the algal diets of the *Elysia*. Here we compare the bacterial communities associated with *E. crispata* from two Caribbean locations with the bacterial communities associated with the algae on which the Puerto Rican *E. crispata* was associated to gain insights into whether the bacterial communities are defined by host-dependency or are primarily derived from the algal diet.

4.3 Experimental methods

4.3.1 Sample collection and processing

E. crispata inhabiting shallow sea grass patches were collected by snorkeling at Little San Salvador, Bahamas (EC2, ECB1, ECB2, and ECB3) (24°34.343'N, 75°56.323'W) in July 2011 and Cayo Enrique, Puerto Rico (EC11, EC21, and EC31) (17°57.313'N, 67°3.193'W) in August 2012. Sacoglossan individuals were placed in separate plastic collection bags filled with surrounding seawater. Individuals were rinsed three times with sterile artificial seawater to remove transiently and loosely attached bacteria. Individuals from the Bahamas were placed in RNAlater (Qiagen Inc., Valencia, CA) within 30 minutes of collection and individuals from Puerto Rico were transferred to sterile Whirl-Pak bags (Fisher Scientific, Pittsburgh, PA) within 30 minutes of collection and immediately stored at -80˚C for molecular analysis. Two of the three *E. crispata* individuals collected in Puerto Rico were found on an unidentified alga and appeared to be feeding on the alga. Two samples of this alga were collected and designated A1 and A2. A portion of the alga associated with *E. crispata* individuals was collected, transferred to sterile Whirl-Pak bags (Fisher Scientific, Pittsburgh, PA), and stored at -80˚C for molecular analysis of the bacterial

communities associated with the alga. Preserved individuals from both locations and the associated alga were later lyophilized prior to extraction of DNA for molecular analysis. Separate individuals of *E. crispata* from Puerto Rico were fixed in 4% formaldehyde for 4 hours and kept in 70% ethanol at 4˚C for microscopy imaging.

4.3.2 Total genomic DNA extraction

Total genomic DNA from lyophilized *E. crispata* was extracted using Mo Bio PowerPlant DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) per manufacturer's instructions.

4.3.3 Cytochrome c oxidase 1 (CO1) gene sequencing and phylogenetic analysis

The CO1 gene from all seven *E. crispata* individuals was PCR amplified from total genomic DNA using forward LCO-1490 and reverse HCO-2198 primers (Folmer *et al*., 1994). DNA amplification of the CO1 gene was performed using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA) in a total reaction volume of 50 µl following the Platinum *Taq* product protocol. Reactions were run in a PTC-200 cycling system (MJ Research, Waltham, MA) using the following cycling parameters: 60 seconds of denaturation at 95˚C, followed by 30 cycles of 30 seconds at 95˚C (denaturing), 30 seconds at 52˚C (annealing), and 30 seconds at 72˚C (elongation), with a final extension at 72˚C for 180 seconds. The PCR-products were sequenced on an ABI 3130 XL Genetic Analyzer (Applied Biosystems, Foster City, CA), using forward LCO-1490 primer. The nearest relatives for each sequence were obtained from the GenBank database using the blastn tool (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analyses were performed with the

MEGA software package (36) and phylogenetic trees were constructed using the neighbor-joining algorithm (37). Bootstrap values were generated using PHYLIP with 1000-replicate data sets.

4.3.4 Mutliplexed 16S rRNA gene sequencing on Illumina MiSeq

Hypervariable regions V4-V5 of the 16S rRNA gene fragments were amplified with primers 515F and 806R. DNA amplification of the 16S rRNA genes was performed in 25 µl reactions with Qiagen HotStar Taq master mix (Qiagen Inc., Valencia, CA), 1 µl of each primer at a concentration of 5 µM and 1 µl of template DNA at a concentration of 20 ng/µl. Reactions were run on a ABI Veriti Thermocycler (Applied Biosystems, Carlsbad, CA) using the following cycling parameters: 300 seconds of denaturation at 95˚C, followed by 35 cycles of 30 seconds at 94˚C (denaturing), 40 seconds at 54˚C (annealing), and 60 seconds at 72˚C (elongation), with a final extension at 72˚C for 600 seconds. Negative controls were included for each amplification and barcoded primer pair, including amplification without template DNA. Amplification products were visualized with eGels (Life Technologies, Grand Island, NY). Products were then pooled equimolar and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, IN) in a 0.7 ratio from both rounds. Size selected pools were then quantified using the Quibit 2.0 fluorometer (Life Technologies, Grand Island, NY) and loaded on an Illumina MiSeq (Illumina Inc., San Diego, CA) 2x300 flow cell at 10 pM by the Research and Testing Laboratory LLC, Lubbock, Texas using protocols recommended by the manufacturer. All 16S rRNA gene amplicons were sequenced as part of the same pool in the same sequencing reaction.

4.3.5 Sequence processing and estimation of bacterial diversity

16S rRNA gene sequences were processed using Mothur (38). Chimeras were removed with UCHIME (39). Sequences were demultiplexed and denoised rigorously as described by Huse *et al*. (40) using Mother. Briefly, sequences were removed from the analysis if they were <200 bp, >275 bp or contained ambiguous characters. Following quality filtration, sequences were clustered at 3% with the average neighbor-joining method. The Bray-Curtis dissimilarity coefficient was used to compare bacterial community structure (41). Bacterial sequences were classified with the RDP classifier (http://rdp.cme.msu.edu/classifier/classifier), while chloroplast sequences were classified with greengenes (http://greengenes.lbl.gov). The nearest relatives for each sequence were obtained from the GenBank database using the blastn tool (http://blast.ncbi.nlm.nih.gov/).

4.3.6 Microscopy and imaging

Following fixation, sea slug samples were placed in 30% sucrose in 1X PBS buffer and left overnight with agitation at 4˚C until tissue sank. Tissues were wiped and embedded in O.C.T (Tissue Tek, Sakura Finetek, Torrance, CA). Thin sections (10 μm) were cut using a microtome (Tissue Tek, Sakura Finetek, Torrance, CA), mounted on glass microscope slides and stained with 4,6-diamidino-2-phenylindole (DAPI 0.1 μg/ml). Images were visualized using epifluorescent microscopy (Zeiss Axioplan, Thornwood, NY).

4.4 Results

4.4.1 Imaging of *E. crispata*

Visualization of *E. crispata* revealed the presence of an associated alga during collection and the presence of chloroplasts harbored intracellularly (Figure 4.1). Microscopic visualization of DAPI-stained sections exposed a diversity of unexplored cells types throughout the right parapodia of the sacoglossan *E. crispata* (Figure 4.1B). These cells are of differing sizes (blue stain), presumably larger eukaryotic and smaller bacterial cells, and colors representing natural autofluorescence (green and red stains). Red fluorescent algal cells were present throughout the parapodia and tightly bound with stained DAPI cells (Figure 4.1C).

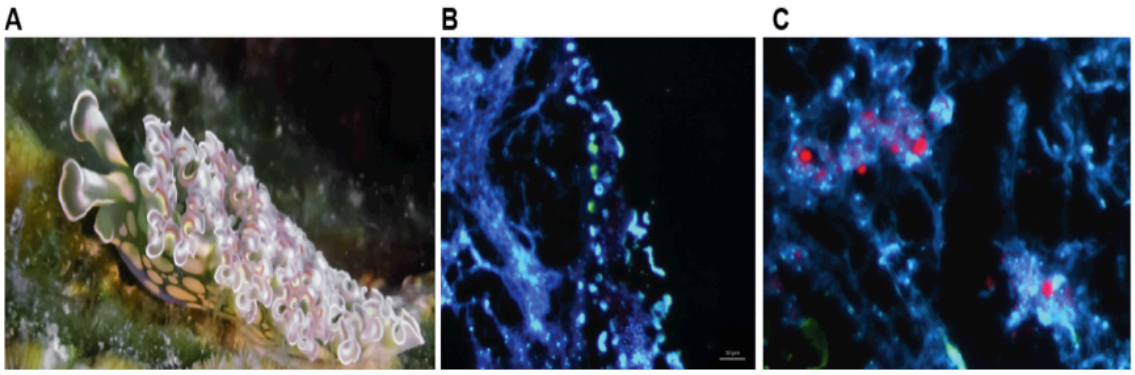


Figure 4.1. Visualization of *E. crispata* associated with unidentified alga. *E. crispata* associated with alga in Puerto Rico during time of collection (A). DAPI images of right parapodia at 10X magnification (B) including ingested chloroplast (red autofluorescence) at 63X magnification (C).

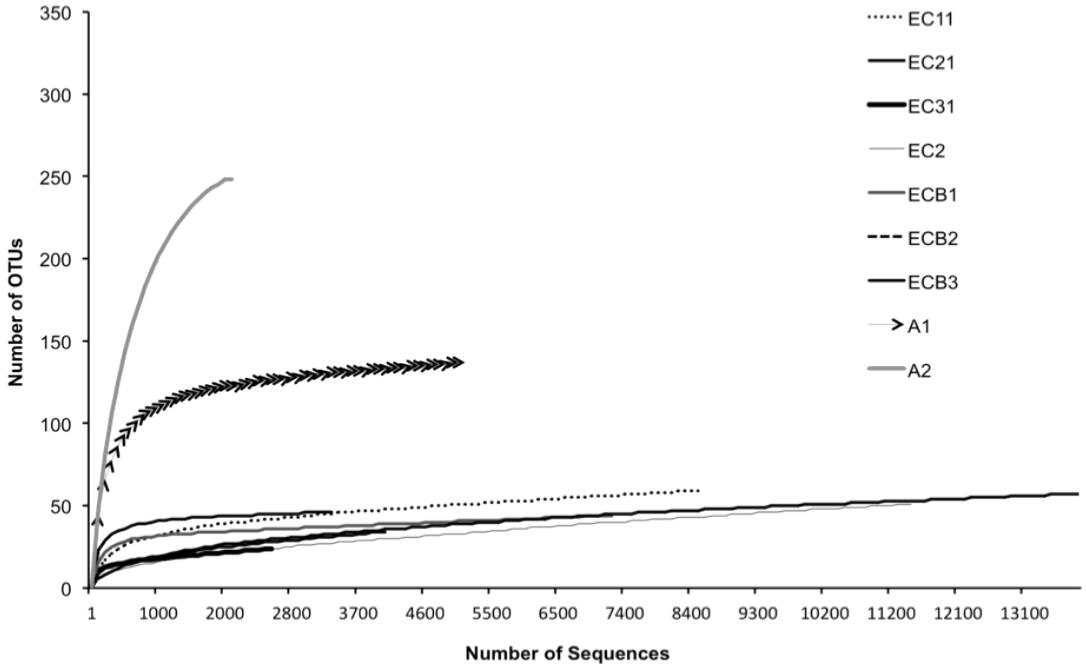
4.4.2 Bacterial community analysis

The *E. crispata* individuals exhibited lower bacterial richness compared to the bacterial communities associated with the alga on which the *E. crispata* were found in Puerto Rico (Table 4.1, Figure 4.2). Rarefaction analysis revealed plateau at around 50 OTUs for the *E. crispata* individuals, indicating that observed OTU richness is well sampled and further sampling of these individuals would generate few new OTUs. The algal samples revealed that further sequencing would be needed to reveal the complete bacterial community associated with the alga. (Figure 4.2). A total of 80,095 16S rRNA gene sequences were analyzed from seven *E. crispata* individuals from two locations in the Caribbean and from the two associated algal samples (Table 4.1). Datasets from the four *E. crispata* individuals from the Bahamas contained 11,700 (EC2), 7,329 (ECB1), 14,279 (ECB2), and 4,340 (ECB3) 16S rRNA gene sequences and together were assigned to 134 OTUs after the removal of 1,036 chloroplast-derived sequences (Table 4.1). Datasets from the three *E. crispata* individuals from Puerto Rico contained 8,460 (EC11), 3,411 (EC21), and 2,790 (EC31) 16S rRNA gene sequences and together were assigned to 108 OTUs after the removal of 222 chloroplast-derived sequences (Table 4.1). The associated algal samples contained 14,646 (A1) and 13,142 (A2) 16S rRNA gene sequences and together were assigned to 335 OTUs after the removal of 20,631 chloroplast-derived sequences.

Table 4.1. Number of 16S rRNA gene sequences analyzed and number of OTUs observed from Caribbean *E. crispata* individuals and associated alga.

Sample	Location	Associated Algae	Sequences			OTUs (bacteria 0.03)
			Bacteria	Chloroplast	Total	
EC2	Bahamas	N	11471	229	11700	51
ECB1	Bahamas	N	7231	98	7329	44
ECB2	Bahamas	N	13849	430	14279	57
ECB3	Bahamas	N	4063	277	4340	34
EC11	Puerto Rico	N	8450	10	8460	59
EC21	Puerto Rico	Y	3369	42	3411	46
EC31	Puerto Rico	Y	2598	192	2790	24
A1	Puerto Rico	___	5128	9518	14646	137
A2	Puerto Rico	___	2027	11113	13140	246
				Total	**80095**	**698**

Figure 4.2. Rarefaction curves of Caribbean sacoglossan individuals and associated alga.



4.4.3 Phylogeny and bacterial composition of *E. crispata*

Analysis of the CO1 gene sequences confirmed the identification of the Caribbean *E. crispata* individuals (Figure 4.3A). For all seven sacoglossan individuals, the closest hit from BLAST analysis was a known *E. crispata* COI gene, with at least 99% identity. Collective analysis of the bacterial 16S rRNA gene sequences revealed a remarkably simple bacterial community associated with these Caribbean slugs, with five phyla representing >96% of the total sequences (Figure 4.3B). The phylum *Verrucomicrobia* was the most abundant bacterial group,

representing 57% of the total sequences. The other major bacterial groups included *Bacteroidetes* (16%), *Alphaproteobacteria* (9%), *Betaproteobacteria* (8%) and *Firmicutes* (6%).

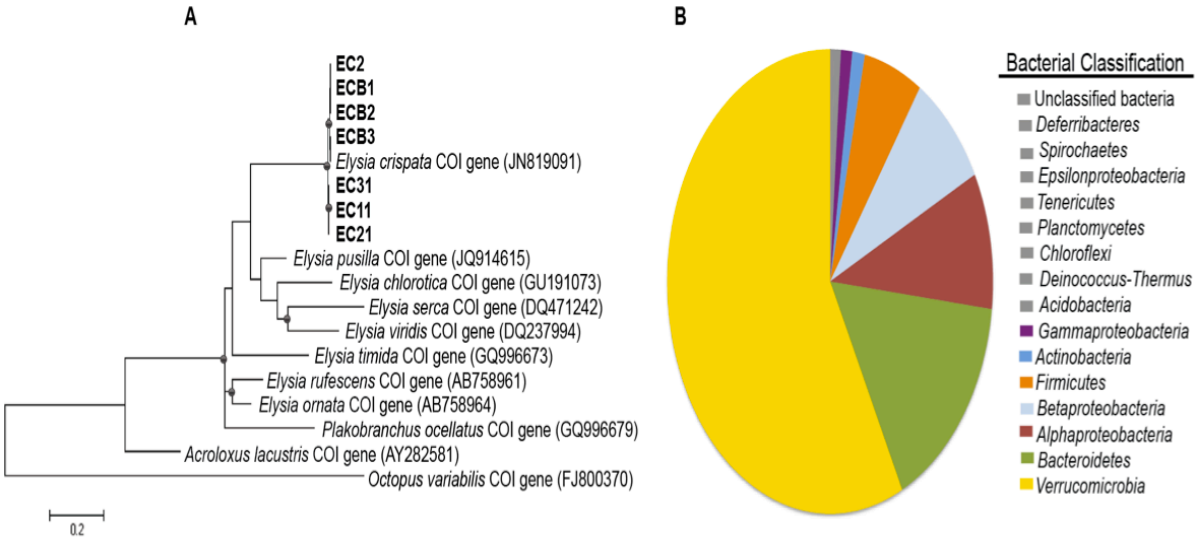


Figure 4.3. Phylogeny and bacterial composition of *E. crispata*. Neighbor-joining tree of CO1 sequences from seven *E. crispata* individuals (bold) and their closest relatives. Bootstraps values (neighbor-joining method, 1000 replicates) are indicated by closed circles (values >60%). The scale bar represents 20% sequence divergence (A). 16S rRNA gene bacterial classification from all sacoglossan individuals. Groups shown in gray represented <1% of total sequences (B).

4.4.4 Bacterial diversity of the Caribbean samples

The *E. crispata* bacterial communities are more similar to each other than to the bacterial community of the associated alga (Figure 4.4). The most dominant bacterial phylum associated with the sacoglossans was *Verrucomicrobia* representing

21% – 87% of the sequences across samples while this phylum represented <1% of the bacterial composition associated with the algal samples. Other abundant bacterial groups associated with *E. crispata* samples included *Betaproteobacteria*, *Firmicutes* and *Bacteroidetes*. Two individuals from Puerto Rico (EC21 and EC31) were slightly different from the other *E. crispata* individuals and varied with respect to the percentage of sequences assigned to bacterial phyla. These two individuals were found associated with the alga and their bacterial communities included the group *Actinobacteria* that was nearly absent in the other slug samples. The algal samples displayed similar bacterial compositions and were dominated by *Alphaproteobacteria* (27% and 37%), *Bacteroidetes* (23% and 36%), and *Gammaproteobacteria* (15% and 19%). All samples were normalized to the least number of sequences across the samples to generate a Bray-Curtis bacterial composition dissimilarity tree between samples.

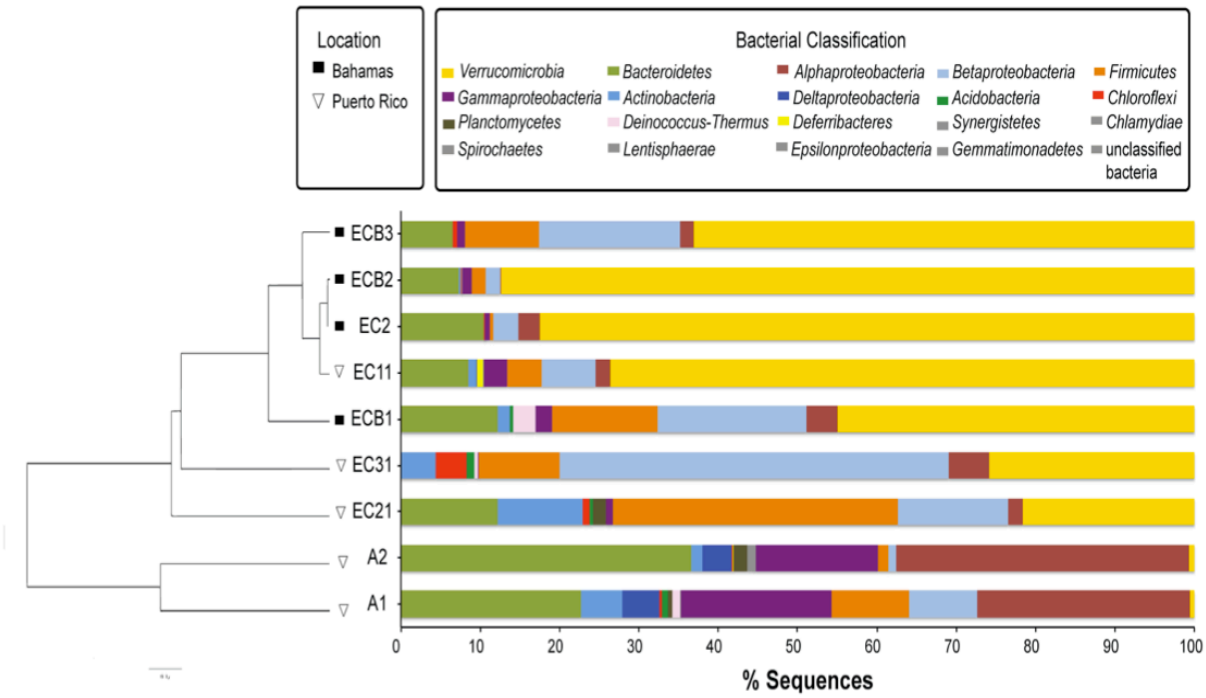


Figure 4.4. Bacterial community similarity and composition across sacoglossan individuals and associated alga. Dendrogram (left) based on Bray-Curtis similarity of bacterial profiles across samples. Host locations are shown as black squares (Bahamas) or inverted triangles (Puerto Rico). Bar charts show the relative abundance of bacterial groups in each sample. Groups shown in gray represented <1% of total sequences. Sequences were normalized to the individual with the least number of sequences.

4.4.5 Diversity of 16S rRNA gene chloroplast-derived sequences

All Caribbean *E.crispata* individuals contained 16S rRNA gene sequences affiliated with those of the chloroplasts from multiple siphonouos green algae of the order *Bryospsidales* (Figure 4.5). The slug individuals from the Bahamas all contained 16S rRNA chloroplast-derived sequences affiliated with *Rhipiliopsis profunda* while all the slug individuals from Puerto Rico retained 16S rRNA chloroplast-derived sequences affiliated with *Bryopsis hypnoides*. Slug individuals in both locations also contained chloroplasts from *Rhipocephalus phoenix* and an unclassified *Ulvophyceae*. The alga found associated with the slugs from Puerto Rico yielded primarily 16S rRNA chloroplast-derived sequences affiliated to those from an unclassified Stramenopile and surprisingly, similar 16S rRNA chloroplast-derived sequences were not affiliated with the slug individuals from Puerto Rico

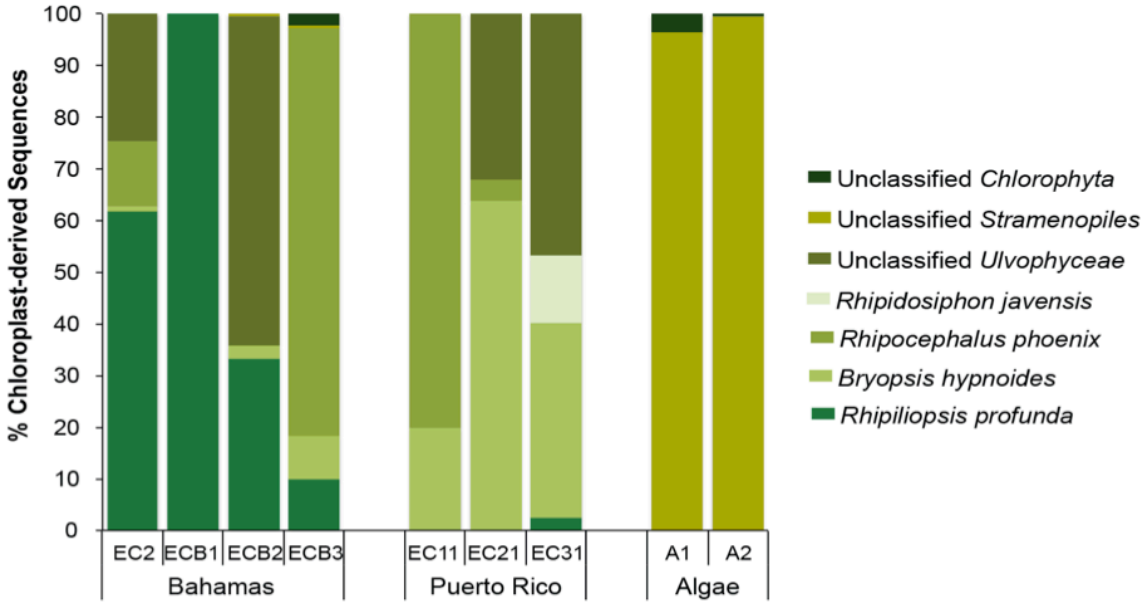


Figure 4.5. Relative abundance of chloroplast-derived sequences in sacoglossan individuals and associated alga.

4.4.6 Shared and core sacoglossan bacterial OTUs

There were a total 12 OTUs shared between the collective Caribbean samples and these OTUs represented 59% of the total sequences (Figure 4.6 and Table 4.2). BLAST analysis of these 12 OTUs revealed 94% – 100% identity to the closest relatives that were isolated from marine sources, plants, the gut of plant-eating insects or the human oral cavity. There were an additional 15 OTUs shared between just the sacoglossan samples most of which were assigned to the phylum Verrucomicrobia (Table 4.3). These OTUs showed remarkable novelty with only 91% – 94% identity to its closet relative isolated from an ascidian. There were 4 OTUs found in all sacoglossan individuals and these OTUs represented nearly 82% of the total sacoglossan sequences (Table 4.4)

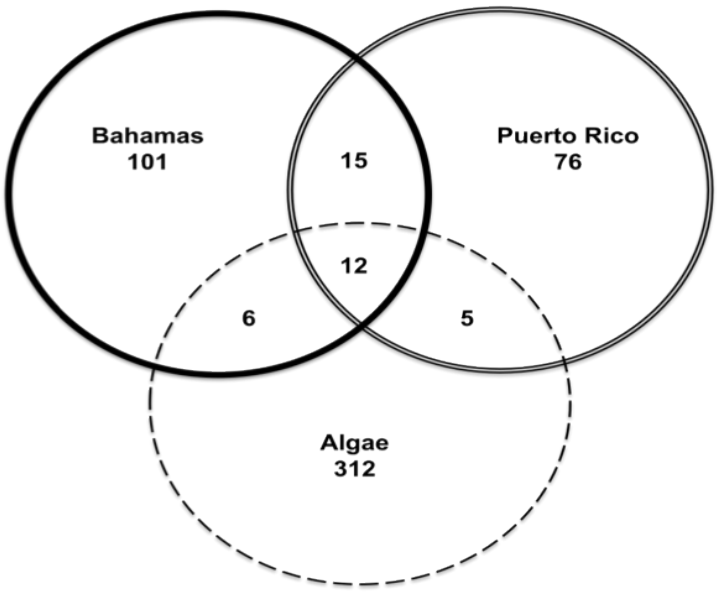


Figure 4.6. Shared OTUs across Caribbean samples. Bahamas and Puerto Rico represent the sacoglossan-derived OTUs found in those locations and alga represent algal-derived OTUs.

Table 4.2. Shared OTUs between Caribbean sacoglossan individuals and the associated alga sample.

OTU	Sequences			Taxonomy	Lowest Taxonomic Rank	BLAST Isolation Source (Identity, Accession #)
	Bahamas	Puerto Rico	Algae			
0001	27298	7295	2	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (94%, AB479420)
0002	2387	2097	402	*β-proteobacteria*	*Delftia* sp.	Insect gut (100%, KJ826597)
0004	2952	1031	6	*Bacteroidetes*	*Ekhidna* sp.	Dinoflagellate (99%, AY701461)
0005	1321	1290	382	*Firmicutes*	*Staphylococcus* sp.	Coral reef (100%, KJ802341)
0014	169	93	14	*α-proteobacteria*	*Bradyrhizobium* sp.	Fabaceae (100%, KJ818095)
0017	202	12	39	*Deinococcus-Thermu*	*Deinococcus* sp.	Soil (97% AY743273)
0023	103	203	2	*β-proteobacteria*	*Ralstonia* sp.	Insect gut (100%, KJ848551)
0035	4	4	5	*Actinobacteria*	*Corynebacterium* sp.	Insect gut (100%, KF115602)
0037	65	13	3	*γ-proteobacteria*	*Pseudomonas* sp.	Insect gut (100%, KJ848516)
0039	6	20	27	*Firmicutes*	*Streptococcus* sp.	Human oral cavity (100%, KF733683)
0422	1	61	70	*Actinobacteria*	*Corynebacterium* sp.	Soil (100, KJ643325)
0487	47	16	15	*Firmicutes*	*Dolosigranulum* sp.	Human oral cavity (100%, AB790156)

Table 4.3. Additional 15 shared OTUs between Caribbean sacoglossan individuals.

OTU	Sequences		Taxonomy	Lowest Taxonomic Rank	BLAST Isolation Source (Identity, Accession #)
	Bahamas	Puerto Rico			
0007	137	40	*γ-proteobacteria*	*Wohlfahrtiimonas* sp.	Ascidian (93%, AB479420)
0010	151	63	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (94%, AB479420)
0015	36	10	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (94%, AB479420)
0016	15	2	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (93%, AB479420)
0018	11	2	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (93%, AB479420)
0020	5	4	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (93%, AB479420)
0025	7	1	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (93%, AB479420)
0026	239	11	*α-proteobacteria*	*Breoghania* sp.	Seafloor (84%, AF468777)
0031	39	106	*Chloroflexi*	*Bellilinea* sp.	*Bryopsis* sp. (99%, JF521615)
0051	5	84	*Actinobacteria*	*Corynebacterium* sp.	Insect gut (100%, KM059075)
0064	35	39	*β-proteobacteria*	*Tepidimonas* sp.	Algal mat (100%, HE817886)
0066	2	1	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (93%, AB479420)
0078	1	1	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (92%, AB479420)
0089	4	3	*Verrucomicrobia*	*Cerasicoccus* sp.	Ascidian (91%, AB479420)
0278	60	62	*α-proteobacteria*	*Roseomonas* sp.	Root plant (99%, KF785588)

Table 4.4. Core sacoglossan OTUs (found in all seven *E. crispata* individuals).

OTU	Caribbean Sacoglossan Individuals							% Sacoglossan Sequences	Phylum
	EC2	ECB1	ECB2	ECB3	EC11	EC21	EC31		
0001	9366	3401	12075	2456	5916	716	663	67.7%	*Verrucomicrobia*
0002	324	1153	196	714	547	420	1130	8.7%	*β-proteobacteria*
0005	8	760	206	347	404	638	248	5.1%	*Firmicutes*
0010	50	23	54	24	56	3	4	0.4%	*Verrucomicrobia*
							Total	**81.9%**	

4.5 Discussion

My study provides the first insights into the bacterial communities associated with the photosynthetic and chemically defended sacoglossan *E. crispata*. Results presented here uncovered that although *E. crispata* and its associated algal food are hosts to many of the same bacterial groups, the bacterial communities associated with *E. crispata* from varying locations are more similar to each other than its associated alga. *Verrucomicrobia* was the most abundant in the bacterial communities associated with the sacoglossans and were rare members of the bacterial community in the associated alga samples. Notably the *Verrucomicrobia*-derived shared OTUs between the sacoglossans displayed 91% – 94% identity to its closest relative, revealing an abundance of a novel, underexplored phylum.

E. crispata harbored a rather simple and low bacterial richness community, compared to the bacterial communities found associated with sacoglossans in other studies. Recent literature shows the bacterial community profiles of the sacoglossans *E. chlorotica* and *E. rufescens* exhibits 4 – 16x and 4 – 12x more OTUs, respectively, than *E. crispata* at the same or lower sampling depths. (Devine *et al*., 2012; Davis *et al*., 2013). One possibility is that the abundance of chemical compounds associated with *E. crispata* selects for a low bacterial rich community compared to other sacoglossans. Chemical compounds associated with ascidians have been known to influence bacterial composition (Wahl *et al*., 1994). Nearly 96% of the total sequences affiliated with the Caribbean sacoglossans were represented by only five phyla. Despite the low bacterial richness, four of the dominant phyla, *Bacteroidetes*, *Alphaproteobacteria*, *Betaproteobacteria,* and *Firmicutes* were also associated with

sacoglossans *E. chlorotica* and *E. rufescens.* Specifically, bacteria in the family Comamonadaceae, which are known to contribute to nitrogen cycling via reduction of nitrate or nitrogen fixation (Khan *et al*., 2002; Sadaie *et al*., 2007; Kampfer *et al*., 2008) were found in previous sacoglossan studies as well as in this study as the dominant member of the total *Betaproteobacteria.* The families Cytophagaceae and Flavobacteriaceae were the dominant members of *Bacteroidetes* in previous sacoglossan studies as well as this study, and bacteria from these groups are often equipped with enzymes capable of degrading various plant polysaccharides such as cellulose and pectin (Kirchman, 2002; Thomas *et al*., 2011). No metabolic functions from bacterial symbionts of *E. crispata* were assessed in this study. However, the presence of specific bacterial groups associated with known microbial profiles of several different *Elysia* spp. indicates they are likely to be important to the sacoglossan hosts and warrants further investigation. As previously mentioned, marine invertebrates are known to form host-specific bacterial communities (Montalvo *et al*., 2011; Dishaw *et al*., 2014).

Verrucomicrobia was the most abundant bacteria group associated with *E. crispata*. *Verrucomicrobia* is a group commonly found in soil bacterial communities (Bergmann *et al*., 2011; Janssen, 2006); however a global study revealed that bacteria belonging to this phylum are nearly ubiquitous in the marine environment across a range of environmental conditions (Freitas *et al*., 2012). In addition to their wide distribution, *Verrucomicrobia* have also been found associated with marine invertebrate hosts. Novel members have been isolated from marine sponges, a sea hare and the gut of a marine clamworm (Yoon *et al*., 2007; Choo *et al*., 2007). The

closest relative of the *Verrucomicrobia*-derived OTUs found shared among the sacoglossans was also isolated from a marine invertebrate. There were only four OTUs found in all sacoglossan individuals and half of those OTUs were associated with *Verrucomicrobia* and represented over 68% of the total sacoglossan sequences. *Verrucomicrobia* was also a member of the bacterial community associated with Hawaiian sacoglossan *E. rufescens* and *Vaucheria litorea*, the algal diet of *E. chlorotica* (Devine *et al*., 2012; Davis *et al*., 2013). Although not much is known about the distribution and diversity of *Verrucomicrobia* in the ocean (Rappé and Giovannoni, 2003), it is apparent that bacteria from this phylum are globally ubiquitous, associated with marine hosts, and their dominance suggests they are important symbionts of sacoglossans. *E. crispata* may be an excellent source for culturing novel *Verrucomicrobia* since the sacoglossans contain a natural enrichment of this phylum. Successful culture of these bacteria would help further our understanding of this underexplored bacterial group.

While most LtR sacoglossans are thought to feed exclusively on a single algal species (Rumpho *et al*., 2001; Marín and Ros, 2004; de Vries *et al*., 2013), recent molecular analyses have revealed that some photosynthetic *Elysia* spp. are capable of feeding on multiple algal diets (Curtis *et al*., 2010; Christa *et al*., 2014). Analysis of the 16S rRNA gene chloroplast-derived sequences revealed that *E. crispata* is not only capable of feeding on various siphonous algae in the Caribbean but also able to maintain the chloroplasts from various algae of the order *Bryospsidales*. However, none of those algae were found associated with the sacoglossan samples during the time of collection. Instead two sacoglossans were found with an associated

unidentified alga in Puerto Rico. Intriguingly, the chloroplast-derived sequences obtained from the associated alga were not present in the sacoglossan samples. One possibility for the absence of associated algal-derived chloroplast sequences in slug samples is that the sacoglossans were not actively feeding but simply associated with the alga as a camouflaging mechanism. The green color acquired by sacoglossan members from sequester chloroplasts is thought to serve as a camouflage (Pelletreau *et al*., 2011; Rumpho *et al*., 2000), when associated with various algae. It is also noted that the presence of a slug on a given macroalga, other than its food source, can occur as a consequence of trial-and-error foraging behavior on its search for food or simply as shelter when exposed to water turbulence (Marín and Ros, 2004; Wagele *et al*., 2011).

Another possible explanation for this finding is that the chloroplasts from the associated alga were immediately degraded upon feeding and not retained. Recent literature suggest that the acquired plastid itself is directly involved in maintenance by bringing along a replenishable supply of essential genes for photosystem II repair and are therefore equipped for longevity while plastids that do not have these genes are immediately degraded (de Vries *et al*., 2013). It has also been recorded that while some LtR species are known to forage on multiple types of algae, they will only retain specific types of chloroplasts during starvation (Curtis *et al*., 2010; Pelletreau *et al*., 2011; Christa *et al*., 2014). Perhaps in the case of *E. crispata*, there is no benefit to retaining the chloroplast of the associated alga. However, both NR and LtR sacoglossans are able to sequester the chloroplast from the same algal diets; therefore, a correlation between the robustness of specific algal plastids and LtR kleptoplasty is

not the sole factor for plastid retention and maintenance (Curtis *et al*., 2010; de Vries *et a*l., 2013). Lastly, the absence of associated algal-derived chloroplast sequences in slug samples could be attributed to the slugs feeding on the alga for chemical compounds rather than chloroplasts. Perhaps these sea slugs already had enough chloroplasts retained for energy, as there were multiple types of chloroplast-derived sequences affiliated with them. Sacoglossans feed on algae that produce various noxious compounds, which serve as feeding deterrents of generalist herbivores (Gallop *et al*., 1980) yet sacoglossans have developed a tolerance for these compounds and are able to harbor them as defense molecules. Several toxic compounds derived from associated algae have been extracted from *E. crispata* from various locations including in the Caribbean (Gavagnin *et al*., 2000).

It is still not clear how sacoglossans maintain chloroplasts, as recent deep sequencing work showed that three different LtR slugs do not express any genes of algal origin (Pelletreau *et al*., 2011; Cruz *et al*., 2013; Bhattacharya *et al*., 2013), revealing that horizontal gene transfer is not the mechanism underlying kleptoplasty survival. The chloroplast types found associated with *E. crispata* in this study do not necessarily imply functional kleptoplasty but confirm the ability of this sacoglossan to feed on and maintain chloroplasts from a variety of algal diets.

To understand the bacterial community associated with a photosynthetic and chemically defended sacoglossan I used 16S rRNA gene deep sequencing analysis to compare the bacterial communities of *E. crispata* from two distinct locations within the Caribbean with an associated alga. I found that the bacterial profiles associated with the sacoglossan individuals from different locations were more similar to each

other than to the bacterial community in one of its associated algae. Importantly, individuals of *E. crispata* from two locations in the Caribbean had bacterial communities heavily dominated by novel representatives of the recently described bacterial phylum *Verrucomicrobia* and in this respect differ from the alga and from other species of *Elysia*.

Chapter 5: Conclusions and future directions

5.1 Conclusions

In this chapter, I reaffirm the research objectives and review the contributions and significance of my work in meeting each objective. I also discuss several questions arising from my data and approaches that can be used to answer these questions.

Previous studies in our laboratory and in collaboration with Dr. Mark Hamann's group at the University of Mississippi suggest that the chemical defense molecule and anticancer metabolite kahalalide F (Becerro *et al*., 2001; Gao and Hamann, 2011) extracted from the sacoglossan mollusk *E. rufescens* and algal diet *Bryopsis* sp. (Hamann and Scheuer, 1993) was produced by an associated bacterium (Hill *et al*., 2003). Previously, extracts from 40 cultured bacteria grown in one medium were screened by high-performance liquid chromatography for the presence of kahalalides. My research significantly expanded on this work by increasing the number of bacteria cultured and screened from the assemblage of bacteria isolated from the sacoglossan *E. rufescens* and the alga *Bryopsis* sp. A total of 460 isolates were screened on several media by using a high-throughput screening assay that I developed. Due to the detection limits in the screening assay and our lack of knowledge of the growth conditions that may be required to stimulate the production of KF, I cannot unequivocally rule out the possibility that an examined cultured bacterium is responsible for KF production, but I did not detect the production of KF by any of the strains that I screened. These results led to a series of broader ecological research goals to better understand the bacterial community associated with sacoglossans. My research examined the bacteria associated with sacoglossan

mollusks in broad terms rather than with a sole focus of isolating a bacterial symbiont that can produce the anticancer metabolite KF.

My research objectives started with the characterization of the total bacterial community associated with *E. rufescens* and its secreted mucus. Sacoglossans often secrete defense molecules into their mucus layer (Marín and Ros, 2004). Chapters 2 and 3 include the bacterial cultivation and deep-sequencing analysis of whole *E. rufescens* and its secreted mucus, as well as the localization of KF in the mollusk through microscopy. I found a highly diverse bacterial community associated with *E. rufescens* in which 16 different bacterial phyla were represented. For the first time we found that KF is localized in the exterior layer of the sacoglossan through collaboration with Dr. Pieter Dorrestein, suggesting its role as a defense molecule in the mucus layer. My phylogenetic analyses of the abundant bacterial groups of *Vibrio* spp. and *Mycoplasma* spp. associated with *E. rufescens* revealed that in most cases the closest relatives of these OTUs were isolated from other mollusks or the mucus layer of corals, and uncultured clones obtained from *Bryopsis* sp. or the gut of other mollusks that are known to forage on algae, respectively. These data suggest that these bacteria are not specialized associates solely of *E. rufescens* and that these bacterial groups may have roles in sacoglossan biology. Results in this study show the complex and diverse groups of bacteria associated with *E. rufescens* that were not evident through standard culturing analysis and suggest that *Vibrio* spp. are symbionts that may play a specific role in the mucus layer. Although it is known that sacoglossans can sequester plastids and chemicals from their algal diets (Rumpho *et*

al., 2001; Avila, 1995), these data for the first time suggest the possible acquisition of bacteria by grazing of the sacoglossans on algae.

My next research focus was to determine the cultivable diversity associated with *E. rufescens* and its algal diet *Bryopsis* sp. and determine potential roles of these bacteria, specifically in the production of KF. This research included isolation of 460 bacterial isolates that I cultured from *E. rufescens* and its algal diet over three years of collection (2010, 2012, and 2014). The identities of a subset of 156 lf those bacteria were determined by 16S rRNA gene sequence analysis. I found that *Vibrio* spp. remain as dominant bacteria over the three years as did *Flavobacteria*, that were specifically associated with *Bryopsis* sp. collected from the sea. I then examined the potential roles of the *Vibrio* spp. in this assemblage by sequencing the genome of a representative *Vibrio* sp. ER1A, as well as screening *Vibrio* spp. for signal molecules (AHLs). The genome sequence of *Vibrio* sp. ER1A showed that it does not possess the genes required to produce KF and that this strain has very few genes that are related to secondary metabolite production. I instead found the presence of specific types of gene clusters that are involved in AHL production, iron acquisition, and biofilm production, which shed light on the potential roles of this bacterium within the sacoglossan host. In addition, I discovered that the highest proportion of AHL molecules was found associated with the *Vibrio* spp. isolated from *E. rufescens* specifically in the mucus layer, where KF has been localized (Davis *et al*., 2013), compared to the *Vibrio* spp. isolated from the surrounding water. These data suggest a potential role of AHLs in colonization and perhaps KF production. These results

further establish *Vibrio* spp. as symbionts of the sacoglossan host, start to assign function to *Vibrio* spp. and imply a beneficial role of this symbiont.

I sought to broaden our understanding of bacterial symbiosis in sacoglossan mollusks by examining the bacterial community of a known photosynthetic and chemically defended sea slug, *Elysia crispata* from two separate Caribbean locations. The bacterial profiles from *E. rufescens* suggest the acquisition of bacteria by algal grazing and therefore the bacterial community of an associated alga of *E. crispata* was also examined. I hypothesized that *E. crispata* and an associated alga would harbor many of the same bacterial groups; however, the bacteria associated with *E. crispata* from two different Caribbean locations would be more similar to each other than to the bacteria found from the associated alga. The results indeed support this prediction. Notably the dominant bacterial groups that were associated with *E. rufescens* from Hawaii, *Vibrio* spp. and *Mycoplasma* spp. were present but rare in the bacterial community of *E. crispata* and its associated alga. I instead found that a novel, underexplored phylum *Verrucomicrobia* is highly abundant in *E. crispata*, although this phylum comprised only less than 1% of the total sequences associated with *E. rufescens*. In addition, I found several groups of bacteria that were not only associated with *E. crispata* from different locations and with *E. rufescens,* but also found by others to be associated with *E. chlorotica* (Devine *et al*., 2012), a photosynthetic slug collected in two locations in the north Atlantic. These results reveal an overlap in the bacterial composition associated with various sacoglossan species from different locations, which demonstrate that these bacteria may be fundamentally important to the unusual sacoglossan-algal niche.

It is important to note that these sea slugs were not only from different locations but different DNA extractions methods were used, yet I was still able to see an overlap in bacterial community compositon among the sacoglassans. Because the overall total bacterial abundance is not known for *E. rufescens* or *E. crispata*, the bacterial groups assessed throughout my analysis represent relative abundance within each sample.

Several research chapters highlighted the concept of sacoglossan mucus-associated bacteria. Although the biochemical composition of the sea slug mucus layer has not been characterized, the mucus layers of other mollusks are characterized by mostly water and the remaining components are lipids and glycoproteins called mucins (Verdugo *et al*., 1987; Davies and Hawkins, 1998). This mucus layer can serve as a protective barrier, facilitate movment, assist in reproduction, and may play a role in communication (Marín *et al*., 1991; Davies and Hawkins, 1998; Chase and Blanchard, 2006; Kuanpradit *et al*., 2012). The human gastrointestinal tract serves as a great model to illustrate how mucus-associated bacteria thrive in a glycan rich environment by producing mucus-degarding enzymes amd mucus-binding extracellular proteins (van Passel *et al*., 2011; Ouwerkerk *et al*., 2013). This is well documented in the genome of several bacteria of the genus *Verrucomicrbia* that are associated with the human intestinal tract (Ouwerkerk *et al*., 2013). A recently discovered species of *Verrucomicrobia* can effectively use human mucus as a carbon and nitrogen source (van Passel *et al*., 2011). The mucus layer is a unique environment both in the chemical composition and potential functional roles. My research suggests mucus-associated bacteria in the molecular and culture-based

analysis of *E. rufescens.* The bacterial groups *Verrucomicrobia*, which are known to degrade mucus and *Vibrio* spp., which are known to participate in signaling and the production of bioactive compounds were found associated with sea slugs samples that harbor copious amounts of mucus.

In combination, these data presented throughout the research chapters reveal the complex bacterial communities associated with two sacoglossans mollusks from different oceans, one of which is chemically defended and harbors a known anticancer compound. By first establishing which bacteria are present in sacoglossans, this dissertation provides insights into the relevant ecological questions that are necessary in understanding the sacoglossan lifestyle. This work highlights the bacterial groups that are conserved in sacoglossans from different environments and are likely to be important symbionts and warrants further investigation. This research adds to the growing understanding of the importance of bacterial symbiosis in the ecology of marine invertebrates.

5.2 Future directions

The research that I have summarized raised a number of questions. Future research needed to address these questions include exploring the bacterial community associated with other sacoglossans, determining the functional or ecological roles of the associated bacterial communities, and identifying the bacterium or bacteria responsible for KF production.

Is there a core or essential bacterial community associated with sacoglossans? First, I define a core bacterial community in two aspects: 1) an assemblage of taxonomically or phylogenetically distinct bacteria shared among sacoglossans or 2)

an assemblage of bacteria with conserved metabolic function shared among sacoglossans; these two properties are of course often linked. This question is both interesting and challenging to answer. Considering the taxonomic and phylogenetic diversity, the range of kleptoplasty, and the varied chemical metabolites associated with these mollusks, it is possible that each bacterial community is tailored to the specific niche of a particular slug species. For example, a slug that is chemically defended may harbor a different bacterial community than one that is not defended, as associated bacteria may play a role in production of the defense compound or the compound itself may possess antimicrobial properties that help shape the bacterial community. For example, marine sponges that have been extensively studied for their microbial symbionts and defense molecules show sponge associated lineages ranging from the species level, *Synechococcus spongiarum* (Usher *et al*., 2004;) to the phylum level, *Poribacteria* (Fieseler *et al*. 2004), regardless of the sponge taxonomy or location (Taylor *et al*., 2007; Montalvo *et al*., 2013). This finding illustrates that a marine invertebrate is capable of harboring host-specific bacteria. I will first propose experiments that would address the possibility of a core sacoglossan bacterial community in terms of shared taxa.

If there is a core group of bacteria present in sacoglossans, I hypothesize that these bacterial taxa would be stable over time and seasonal changes. One experiment that would provide insights would be to assess by high-throughput 16S rRNA gene sequencing the bacterial community associated with the sacoglossan mollusks *E. rufescens* and *E. crispata* over a multi-year time course with seasonal variation. These sacoglossans are distantly related, demonstrate differing abilities in plastid

uptake and maintenance, and display different chemical metabolites, making them attractive candidates for continued bacterial community analysis. From my research it is now apparent that there are some similarities in the bacterial communities associated with these ecologically distinct sacoglossans however, these data are limited to one year of sampling. In addition, research should include other phylogenetically distinct sacoglossans from various locations and environments. I expect that host-specific bacterial groups would be not only stable over time but also found in various sacoglossan taxa in different locations.

The proposed work focuses on bacterial community studies of sacoglossans sampled in their natural habitat. However, several sacoglossan slugs can be maintained in captivity (Pierce and Curtis, 2012). If laboratory conditions closely replicate natural environmental parameters such as salinity, temperature, light exposure, etc., I hypothesize that core bacterial taxa will be maintained even under artificial laboratory conditions. Therefore one promising step in determining a core bacterial community would be to assess the bacterial community of laboratory-bred sacoglossans. It is expected that the bacterial community may shift upon transfer to aquaculture, as the conditions of their native ecological environment cannot be perfectly replicated. However monitoring the community over time may reveal groups of bacteria that are consistently present.

It is possible that some of the bacteria found associated with sacoglossan hosts are loosely attached bacteria from the water column. I hypothesize that the core bacterial community, including any essential bacteria, will be metabolically active under observed conditions and present at low abundance and with low or absent

metabolic activity in the surrounding seawater. In addition to the experiments described above, it would be revealing to characterize the active bacterial community (16S rRNA gene profiling from total community RNA) with the *Elysia* and the surrounding seawater samples being assessed in parallel with each study.

Lastly, I hypothesize that within the potential core bacterial groups found among sacoglossans are some bacteria that are vertically transferred through sacoglossan generations. To test this, fluorescence in situ hybridization (FISH) and sequencing approaches could be used to detect putative core bacteria in sacoglossan larvae. Given that core bacterial symbionts may add to the fitness of their host, maintenance through vertical transmission is possible and should be explored.

Once putative core bacterial groups are identified, phylogenetic analysis can be used to identify taxonomically distinctive bacterial clusters. It is possible that these experiments may not lead to a discovery of new bacterial phyla or genera only found associated with sacoglossans. It is perhaps more likely the 16S rRNA gene sequences retrieved from these sacoglossan community analyses will be more similar to each other than to any other available 16S rRNA gene sequences in the database, exposing possible sacoglossan-specific clusters within already-known bacterial taxa. My research on the phylogenetic analysis of the *Vibrio* spp. associated with *E. rufescens* shed light on this concept, as their closest relatives were isolated from other mollusks. This illustrates that even ubiquitous bacteria can form clusters that are unique to invertebrate hosts. My research on the bacterial profile associated with *E. crispata* revealed an abundant novel clade of *Verrucomicrobia* in *E. crispata* individuals that was distinct from other known *Verrucomicrobia* clades, further

suggesting distinctive host-specific clusters. Several reports have demonstrated that wood-eating termites harbor a phylogenetically diverse assemblage of *Verrucomicrobia* within their guts that are distinct from other known *Verrucomicrobia* lineages (Stevenson *et al*., 2004; Wertz *et al*., 2012).

Although it is challenging to prove that there is a core bacterial community associated with sacoglossans the proposed experiments should be successful in determining the presence of such a community. These studies will also reveal variability in associated bacterial communities and could identify potential bacterial sub-populations in relation to sacoglossan phylogeny and sampling location. A fundamental taxonomic core community could emerge with a distinct phylogeny that is unique to sacoglossan hosts. A core bacterial group found in several sacoglossan species, would suggest that ancestral bacterial strains might have first inhabited a sacoglossan prior to slug speciation and are maintained irrespective of host phylogeny, geographic location, or environmental conditions.

Although 16S rRNA gene sequences do not necessarily reveal functional metabolic potential, ecological patterns revealed through such analysis can provide significant insights into the mechanisms influencing community composition. Phylogenetic relatedness among bacterial strains with known ecological functions in a community can be used as a guide to test possible functional roles in a given host community. Therefore once the core bacterial taxa are identified in different sacoglossan hosts under various conditions, the next step is to assign functional roles to those bacteria. I will now discuss experiments that will address a core sacoglossan bacterial community in terms of shared ecological function.

In spite of the phylogenetic diversity and various environmental niches, sacoglossan mollusks feed on algae, which often contain distinct sugars. Therefore, I hypothesize there is a core group of bacteria responsible for algal degradation. One testable approach would be functional gene-targeted metagenomics of cellulases required for algal degradation on various sacoglossan samples. My research revealed members of *Bacteroidetes*, which are often equipped with enzymes capable of degrading various plant materials (Kirchman, 2002; Thomas *et al*. 2011), associated with both *E. rufescens* and *E. crispata* and their associated algae, and also found by others to be associated with the sacoglossan *E. chlorotica* (Devine *et al*., 2012). Members of *Bacteroidetes* have developed mechanisms to utilize niche-specific polysaccharides (Thomas *et al*. 2011), therefore enzymes needed to breakdown plant sugars are a targeted function for this phylum. Another approach includes whole genome "shotgun" metagenomics of various sacoglossans to assess enzymes capable of degrading plant materials. This approach does not require PCR amplification of genes related to algal degradation. Bacterial cells can be separated from host cells prior to genomic preparation for the metagenomic approach to specifically target bacterial DNA and reduce contaminating host DNA.

Sacoglossans have two known pathways for obtaining carbon substrates: 1) through algal grazing and/or 2) through photosynthesis by ingested chloroplasts. Such conditions of a high supply of carbon could rapidly lead to nitrogen deprivation. I hypothesize that there is a core bacterial group responsible for nitrogen acquisition. The experiments required to test this hypothesis are as previously mentioned both functional gene-targeted metagenomics and whole genome metagenomics. In this

case genes required for nitrogen fixation and nitrogen cycling should be targeted. My research on the bacterial community associated with *E. rufescens* and *E. crispata* revealed several members known to play a role in nitrogen acquisition in other hosts such as members of *Alphaproteobacteria* and *Betaproteobacteria*. In addition, members of the phyla *Verrucomicrobia* are known to fix nitrogen (Wertz *et al*., 2012) and were found in association with both studied *Elysia* spp.

The described metagenomics technologies have both advantages and limitations. Whole genome metagenomics allows for a robust representation of the bacterial community since it is not biased by PCR amplification of a single gene; however, all genes may not be sequenced equally with this approach and it may be challenging to accurately infer the bacterial origin from the metagenomic reads (Poretsky *et al*., 2014). On the other hand, gene-targeted metagenomics can provide accurate insights into the bacterium responsible for the function and can often provide insights into rare bacterial diversity not detected by whole genome metagenomics (Kembel *et al*., 2011). Therefore, these two approaches should be combined to assess a functional core of bacteria. These approaches could be extended to include metatranscriptomics, which would enable assessment of the active bacterial community associated with sacoglossans.

Metagenomics allows for the evaluation of the bacterial community composition along with functional gene diversity without the need to amplify specific genes and this approach is of course not limited to questions of plant polysaccharide degradation or nitrogen acquisition. Metagenomics with sufficient sequencing depth could answer several ecological questions related to the sacoglossan lifestyle.

Currently the mechanisms by which sacoglossans store chloroplasts and photosynthesize is not known and deep sequencing analyses from three of the six known photosynthetic slugs did not reveal any genes of algal origin (Pelletreau *et al*., 2011; Wägele *et al*., 2011; Bhattacharya *et al*., 2013). Is there a core group of bacteria and/or functional genes that are in abundance during times of chloroplast uptake and maintenance? Since chloroplasts are not transferred to larvae and must be acquired afresh by each generation (Rumpho *et al*. 2001; Bhattacharya *et al*., 2013), a metagenomics approach could be used to assess the bacterial profile and gene diversity during initial plastid uptake. Analysis should include assessment of the larval stage up until metamorphosis when the first green pigments appear. All six of the known photosynthetic slugs have been maintained and studied in captivity. These photosynthetic animals therefore serve as useful models for studying coevolved relationships between host, their plastids, and associated microbiota. My imaging analysis of *E. crispata* revealed small cells, presumably bacteria, tightly bound around algal cells, illustrating a possible biochemical role performed by bacteria that aids in chloroplast maintenance in a foreign host. Recent data revealed that some acquired plastids come equipped with genes that aid in longevity. However those genes are not present in acquired plastids of *Bryopsis* sp. (de Vries *et al*., 2013) and therefore these plastids should not display long-term maintenance in the sacoglossan hosts. My research of the chloroplast-derived 16S rRNA genes associated with *E. rufescens* and *E. crispata* revealed the feeding of both these slug species on *Bryopsis* sp. *E. rufescens* has not been confirmed to photosynthesize whereas *E. crispata* has shown to photosynthesize. Therefore acquiring plastids that are equipped for

longevity does not necessarily lead to photosynthesis. Perhaps chemicals produced by associated bacteria protect acquired plastids from photo damage and allow for longevity.

Many sacoglossans can be studied in captivity and thus several studies can be conducted to better understand the ecology of sea slugs. Additional questions include: how do bacterial community profiles change when the sacoglossans are starved? Does the bacterial community change when sacoglossans feed on different sources of algae? Do bacteria play a role in metamorphosis from shelled larvae to shell-less slugs? It is widely accepted that sacoglossans do not metamorphose without the presence of their preferred algal food (Rumpho *et al*., 2011); therefore metagenomics could be used to determine whether there is there a core group of bacteria that enable metamorphosis. It is important to understand how environmental factors and stresses affect the composition and function of bacteria and the overall outcome of host-microbe interactions. Metagenomic tools can be used to answer these broad ecological questions about the dynamic lifestyle of sacoglossans and once certain bacteria or genes are revealed to be important, they can be the focus for further studies. Future experiments are proposed in Figure 5.1.

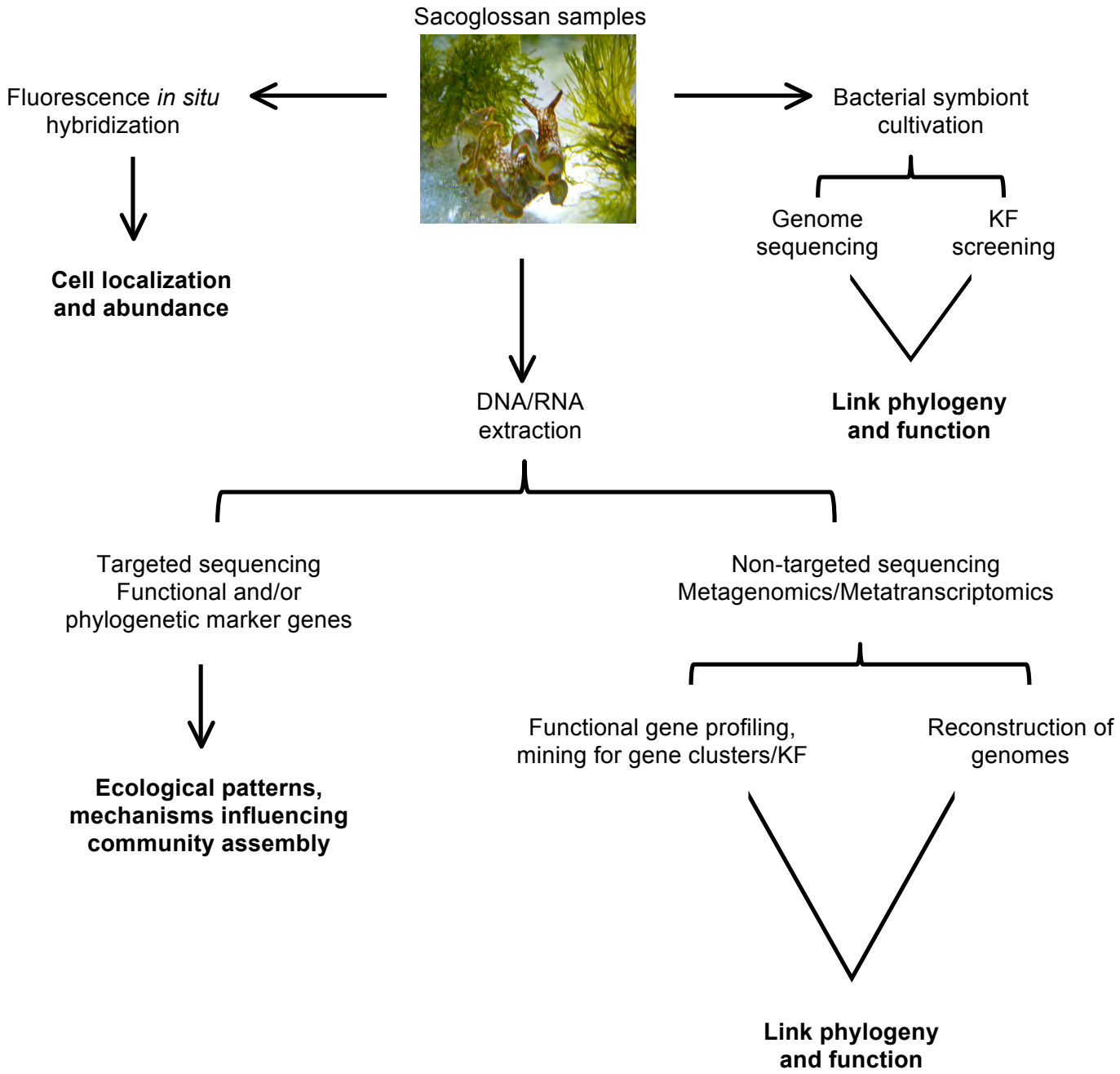


Figure 5.1. Summary of molecular approaches needed for the study of bacterial symbionts in sacoglossan hosts. The benefits of each approach are bolded.

Although determining ecological roles of bacteria is challenging, metagenomic and metatranscriptomic approaches are the most direct means to examine the ecological functions of the sacoglossan bacterial community and link patterns of function to patterns of community composition. This helps getting at the question of a core bacterial community. Given the relative ease at which bacteria can exchange genetic material, it is possible that several groups of bacteria play the same functions in a community. Therefore, it is likely that the bacterial communities associated with these sacoglossans are structured based on function rather than composition only.

Is a bacterium responsible for the production of KF? Bacteria grow in consortia and the metabolism of one species can result in the production of compound a or compounds in another species. Slater and Bull (1982) revealed a six membered bacterial consortia from soil in which the herbicide was broken down from the co-metabolism of four bacteria whereas the other two bacteria played no role in herbicide degradation but simply existed from the by-products of the initial four species. This is a good demonstration of the fact that bacteria have concerted interactions. My extensive attempts to culture a wide diversity of bacteria and to screen these isolates for KF-producers were unsuccessful in identifying a KF-producer. Other culture-based approaches could be taken to attempt to isolate KF-producing bacteria, for example, approaches that allow for metabolite exchange by growing the cultured bacteria in diffusion chambers in various co-culture configurations. Screening KF could be by antifungal assays or by MS.

Our research collaboration with Dr. Dorrestein revealed the localization of KF through MALDI imaging in the exterior layer of *E. rufescens* where copious amounts of mucus is secreted (Davis *et al*. 2013). This data is consistent with the ecological role of KF as it is a chemical defense molecule, and molecules of this type are often secreted in the mucus layer of sacoglossans (Marín and Ros, 2004). In addition, the AHL screening of cultured *Vibrio* spp. revealed the highest proportion of AHLs positive bacteria were isolated from the secreted mucus layer of *E. rufescens*. Based on the research presented an appropriate question is: do QS molecules play a role in the production of KF? One approach to explore this possibility is to add AHLs directly to the media during KF screening. Perhaps at the right concentration these signaling molecules could induce in the production of KF in cultured isolates. The same technique could be used for other signaling molecules such as cyclic adenosine monophosphate (cAMP). Research has shown that both cAMP and AHL signaling molecules have aided in cultivation efficiency (Bruns *et al*. 2002) and these molecules could potential serve as helper molecules in metabolite production. Limitations to this approach include the fact that signaling molecules can be unstable under certain media conditions and that other bacteria can selectively destroy signaling molecules (Tait *et al*. 2009), making it difficult to find optimal conditions under which these molecules would induce KF production. However, given the importance of QS in the production of secondary metabolites (Williams *et al*., 2007), more considerations should be given exploring the role of these signaling molecules in KF production. Ultimately, bacteria may require specific chemical substrates to produce specific compounds. The use of the diffusion chambers that allow for the

passage of chemicals found in the natural environment as well as the addition of known signaling molecules could help in finding the KF producing bacterium.

As previously discussed, whole genome metagenomics can be used to find specific genes of interest without gene amplification. A very attractive direct approach to determine whether a bacterium is responsible for KF production is deep metagenomic sequencing of the total genomic DNA (bacterial and host) extracted from *E. rufescens* and its diet *Bryopsis* sp., both of which are known to contain KF. This would be followed by bioinformatics analysis to attempt to locate the biosynthetic genes that are responsible for the production of KF. Based on the chemical structure, it has been predicted that KF is produced by a nonribosomal peptide synthetase, an enzyme known to produce peptides that are cyclic and/or branched in structure and are linked amino acids (as in the structure of KF). The adenylation (A) domain is required and selects the cognate amino acid from the pool of available substrates and thus it activates the first amino acid and allows for the loading of amino acids to a growing chain (Marahiel *et al*. 1997). Searching the metagenome for specific A domains that correspond to the amino acids in KF is a direct way to find the biosynthetic gene cluster encoding KF.

A metagenomic approach along these line was very recently performed by Dr. Mohamed Donia on samples provided through a collaboration with the Hill and Hamann Laboratories. In an exciting finding by Dr. Donia, metagenomic sequencing of total DNA extracted from *Bryopsis* sp. followed by his bioinformatics analysis has provided preliminary data that strongly indicate that a *Flavobacterium* sp. is responsible for the production of KF. This data is consistent with my research, in

which I cultured diverse *Flavobacteria* from *Bryopsis* sp. and found the presence of *Flavobacteria* in *E. rufescens* secreted mucus by deep sequencing analysis of 16S rRNA genes. The preliminary research findings of Dr. Donia are currently undergoing confirmation.

My dissertation characterizes the bacterial community associated with two distinct sacoglossan mollusks and their associated algae. I used an integrated approach of culture-based and culture independent analysis and for the first time provide information on the ecological patterns of bacteria associated with sacoglossans. I began to assign potential functional roles of these bacteria. My work lays a substantial foundation for future experiments that are needed to advance the field of bacterial symbiosis in sacoglossan hosts. My research helps better our understanding of the interesting ecology of sacoglossan slugs and their associated bacteria.

Literature cited

Abdallah, F.B., Ellafi, A., Lagha, R., Kallel, H., and Bakhrouf, A. (2011) Virulence gene expression, proteins secreted and morphological alterations of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* in response to long-term starvation in seawater. *Afr J Microbiol Res* **5:** 792-801.

Adolph, S., Jung, V., Rattke, J., and Pohnert, G. (2005) Wound closure in the invasive green alga *Caulerpa taxifolia* by enzymatic activation of a protein crosslinker. *Angew Chem* **44:** 2-4.

Alagely, A., Kredlet, C.J., Ritchie, K.B., and Teplitski, M. (2011) Signaling-mediated cross-talk modulates swarming and biofilm in a coral pathogen *Serratia marcescens*. *ISME J* **5:** 1609-1620.

Armstrong, E., Yan, L., Boyd, K.G., Wright, P.C., and Burgess, J.G. (2001) The symbiotic role of marine microbes on living surfaces. *Hydrobiologia* **461**: 37.

Avila, C. (1992) A preliminary catalogue of natural substances of opisthobranch molluscs from western Mediterranean and near Atlantic. *Sci Mar* **56:** 373-382.

Avila, C. (1995) Natural products of opisthobranch molluscs: A biological review. *Oceanography and Marine Biology: Annual Review* 1995 **33:** 487-559.

Azam, F. (1998) Microbial control of oceanic carbon flux: the plot thickens. *Science* **280:** 694-696.

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A. *et al*. (2008) The RAST Server: Rapid annotations using subsystems technology. *BMC Genomics* **9:** 75 doi: 10.1186/1471-2164-9-75.

Bano, N., DeRae, S.A., Bennett, W., Vasquez, L., and Hollibaugh, J.T. (2007) Dominance of *Mycoplasma* in the guts of the Long-Jawed Mudsuker, *Gillichthys mirabilis*, from five California salt marshes. *Environ Microbiol* **9:** 2636-2641.

Beals, E.W. (1984) Bray-Curtis ordination: an effective strategy for analysis of multivariate ecological data, p 1-55. *In* MacFadyen A, Ford ED (ed), Advances in Ecological Research, vol 14, Academic Press, London.

Becerro, M.A., Goetz, G., Paul, V.J., and Scheuer, P.J. (2001) Chemical defenses of the sacoglossan mollusk Elysia rufescens and its host alga *Bryopsis* sp. *J Chem Ecol* **27:** 2287-2299.

Benkendorff, K. (2010) Molluscan biological and chemical diversity: secondary metabolites and medicinal resources produced by marine molluscs. *Biol Rev Camb Philos Soc* **85:** 757-775.

Bergmann, G.T., Bates, S.T., Eilers, K.G., Lauber, C.L., Caporaso, J.G., Walters, W.A., *et al*. (2011) The under-recognized dominance of Verrucomicrobia in soil bacterial communities. *Soil Biol Biochem* **43:** 1450-1455.

Bernhard, J. M., and Bowser, S.S. (1999) Benthic foraminifera of dysoxic sediments: chloroplast sequestration and functional morphology. *Earth-Science Reviews* **46:** 149-165.

Bhattacharya, D., Pelletreau, K.N., Price, D.C., Sarver, K.E., and Rumpho, M.E. (2013) Genome analysis of *Elysia chlorotica* egg DNA provides no evidence for horizontal gene transfer into the germ line of this kleptoplastic mollusc. *Mol Biol Evol* **30:** 1843-1852.

Blackman, A.J., and Wells, R.J. (1978) Flexilin and trifarin, terpene 1,4-diacetoxybuta-1,3 dienes from two *Caulerpa* species (Chlorophyta). *Tetrahedron Lett.* **1978:** 3063-3064.

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., *et al*. (2013) antiSMASH 2.0 – a versatile platform for genome mining of secondary metabolite producers. *Nuc Acids Res* **41:** W204-W212.

Bosch, T.C.G. (2012) Understanding complex host-microbe interactions in *Hydra*. *Gut Microbes* **3:** 345-351.

Bosch, T.C.G., and McFall-Ngai, M. J. (2011) Metaorganisms as the new frontier. *Zoology (Jena)* **114:** 185-90.

Bourne, D., Iida, Y., Uthicke, S., and Smith-Keune, C. (2008) Changes in coral-associated microbial communities during a bleaching event. *ISME J* **2:** 350-363.

Brandt, K. (1883). Über die morphologische und physiologische bedeutung des chlorophylls bei tieren. *Mit Zool Stn Neapel* **4:** 191-302.

Bruns, A., Cypionka, H., and Overmann, J. (2002) Cyclic AMP and acyl homoserine lactones increase the cultivation efficiency of heterotrophic bacteria from the central Baltic Sea. *Appl Environ Microb* **68:** 3978-3987.

Caporaso, J.G, Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., *et al*. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nature Methods* **7:** 335-336.

Carmona, L., Malaquias, M.A.E., Gosliner, T.M., Pola, M., and Cervera, J.L. (2011) Amphi-atlantic distributions and cryptic species in sacoglossan sea slugs. *J Mollus Stud* **77:** 401-412.

Casadevall, A., and Pirofski, L. (2000) Host-pathogen interactions: the basic concept of microbial commenalisms, colonization, infection and disease. *Infect Immun* **68:** 6511-6518.

Casadevall, A., and Pirofski, L. (2001) Host-pathogen interactions: the attributes of virulence. *J Infect Dis* **184:** 337-344.

Casadevall, A., and Pirofski, L. (2014) Ditch the term pathogen. *Nature* **516:** 165-166.

Cavanaugh, C.M., McKiness, Z.P., Newton, I.L.G., and Stewart, F.J. (2006) Marine chemosynthetic symbiosis, p 475-507. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K, Stackebrandt E (ed), The prokaryotes, 3rd ed, vol 1. Springer, New York.

Cavas, L., Baskin, Y., Yurdakoc, K., and Olgun, N. (2006) Antiproliferative and newly attributed apaptotic activities from an invasive marine alga: *Caulerpa racemosa* var. *cylindracea*. *J Exp Mar Biol Ecol* **339:** 111-119.

Chase, R., and Blanchard, K.C. (2006) The snail's love-dart delivers mucus to increase paternity. *Proc Biol Sci* **273:** 1471-1475.

Choo, Y.J., Lee, K., Song, J., and Cho, J.C. (2007) *Puniceicoccus vermicola* gen. nov. sp. nov. a novel marine bacterium, and description of *Puniceicoccaceae* fam. nov. *Puniceicoccales* ord. nov. *Opitutaceae* fam. nov. *Opitutales* ord. nov. and *Opitutae* classis nov. in the phylum '*Verrucomicrobia*'. *Int J Syst Evol Microbiol* **57:** 532-537.

Christa, G., Händeler, K., Schaberle, T.F., Konig, G.M., and Wägele, H. (2014) Identification of sequestered chloroplasts in photosynthetic and non-photosynthetic sacoglossan sea slugs (Mollusca, Gastropoda). *Front Zool* **11:** 15 doi:10.1186/1742-9994-11-15.

Ciavatta, M.L., Gresa, M.P.L., Gavagnin, M., Manzo, E., Mollo, E., DeSouza, L., *et al*. (2006) New caulerpenyne-derived metabolites of an *Elysia* sacoglossan from the south Indian coast. *Molecules* **11:** 808-816.

Cimino, G., Fontana, A., and Gavagnin, M. (1999) Marine opisthobranch molluscs: Chemistry and ecology in sacoglossans and dorids. *Curr Organic Chem* **3:** 327-372.

Cimino, G., and Ghiselin, M.T. (1998) Chemical defense and evolution in the Sacoglossa (Mollusca: Gastropoda: Opisthobranchia). *Chemoecology* **8:** 51-60.

Cimino, G., Passeggio, A., Sodano, G., Spinella, A., and Villani, G. (1991) Alarm pheromones from the Mediterranean opisthobranch *Haminoea navicula*. *Experientia* **47:** 61-63.

Clark, K.B., and Busacca, M. (1978) Feeding specificity and chloroplast retention in four tropical ascoglossa, with a discussion of the extent of chloroplast symbiosis and the evolution of the order. *J Moll Stud* **44:** 272-282.

Clark, K.B., Jensen, K.R., and Stirts, H.M. (1990) Survey for functional kleptoplasty (chloroplast symbiosis) among West Atlantic Ascoglossa (Sacoglossa) (Mollusca: Opisthobranchia). *Veliger* **33:** 339-345.

Coronada, C., Galmarini, C.M., Alfaro, V., and Yovine, A. (2010) Elisidepsin. *Drugs Fut* **35:** 10.1358/dof.2010.035.04.1497635

Costa, A. (1867) Illustrazione di due generi di Molluschi Nudibranchi. *Rendi Accad Sci Fis Mat Napoli* **6:** 136-137.

Craig, A.G. (2000) The characterization of conotoxins. *J Toxicol-Toxin Rev* **19:** 53-93.

Cruz, S., Calado, R., Serôdio, J., and Cartaxana, P. (2013) Crawling leaves: photosynthesis in sacoglossan sea slugs. *J Exp Bot* **64:** 3999-4009.

Cummins, S.F., Nichols, A.E., Schein, C.H., and Nagle, G.T. (2006) Newly identified water-borne protein pheromones interact with attractin to stimulate mate attraction in *Aplysia*. *Peptides* **27:** 597-606.

Curtis, N.E., Schwartz, J.A., and Pierce, S.K. (2010) Ultrastructure of sequestered chloroplasts in sacoglossan gastropods with differing abilities for plastid uptake and maintenance. *Invert Biol* **129:** 297-308.

Daniels, R., Vanderleyden, J., and Michiels, J. (2004) Quorum sensing and swarming in bacteria. *FEMS Microbiol Rev* **28:** 261-289.

Das, S., Lyla, P.S., and Khan, S.A. (2006) Marine microbial diversity and ecology: importance and future perspectives. *Curr Sci* **90:** 1325-1335.

Davidson, S.K., Allen, S.W., Lim, G.E., Anderson, C.M., and Haygood, M.G. (2001) Evidence for the biosynthesis of bryostatins by the bacterial symbiont *Candidatus*

Endobugula sertula of the bryozoan *Bugula neritina*. *Appl Environ Microbiol* **67:** 4531-4537.

Davis, A.R., Benkendorff, K., and Ward, D.W. (2005) Responses of common SE Australian herbivores to three suspected invasive *Caulerpa* spp. *Mar Biol* **146:** 859-868.

Davis, J., Fricke, W.F., Hamann, M.T., Dorrestein, P.C., Esquenazi, E., and Hill, R.T. (2013) Characterization of the bacterial community of the chemically defended Hawaiian sacoglossan *Elysia rufescens. Appl Environ Microbiol* **79:** 7073-7081.

Davies, M.S., and Hawkins, S.J. (1998) Mucus from marine molluscs. *Adv Mar Biol* **34:** 1-71

De Bary, A. (1879) Die ercheinung der symbiose. Strasbourg Turbner KJ

De Negri, A., and De Negri, G. (1876) Farbstoff aus *Elysia viridis*. *Ber Deut Chem Gesellsch* **9:** 84.

Derby, C.D., Kicklighter, C.E., Johnson, P.M., and Zang, X. (2007) Chemical composition of inks of diverse marine molluscs suggests convergent chemical defenses. *J Chem Ecol* **33:** 1105-1113.

Devi, P., Wahidullah, S., Rodrigues, C., and Souza, L.D. (2010) The sponge-associated bacterium *Bacillus licheniformis* SAB1: a source of antimicrobial compounds. *Mar Drugs* **4:** 1203-1212.

Devine, S.P., Pelletreau, K.N., and Rumpho, M.E. (2012) 16S rDNA-based metagenomic analysis of bacterial diversity associated with two populations of the kleptoplastic sea slug *Elysia chlorotica* and its algal prey *Vaucheria litorea*. *Biol Bull* **223:** 138-154.

de Vries, J., Habicht, J., Woehle, C., Changjie, H., Christa, G., Wägele, H., *et al*. (2013) Is ftsH the key to plastid longevity in sacoglossan slugs? *Genome Biol Evol* **5:** 2540-2548.

Dishaw, L.J., Flores-Torres, J., Lax, S., Gemayel, K., Leigh, B., Melillo, D., *et al*. (2014) The gut of geographically disparate *Ciona intestinalis* harbors a core microbiota. *PLoS One* **9**: e93386. doi:10.1371/journal.pone.0093386.

Dobretsov, S., Dahms, H.U., Harder, T., and Qian, P.Y. (2006) Allelochemical defense against epibiosis in the macroalga *Caulerpa racemosa* var. *turbinata*. *Mar Ecol Prog Ser* **318:** 165-175.

Donachie, S.P., and Zdanowski, M.K. (1998) Potential digestive function of bacteria in krill, *Euphausia superba* stomach**.** *Aquat Microb Ecol* **14:** 129-136.

Dubilier, N., Bergin, C., and Lott, C. (2008) Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6:** 725-740.

Dumay, O., Pergent, G., Pergent-Martini, C., and Amade, P. (2002) Variations in caulerpenyne contents in *Caulerpa taxifolia* and *Caulerpa racemosa. J Chem Ecol* **28:** 343-352.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., and Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27:** 2194-2200.

Elston, R.A., Hasegawa, H., Humphrey, K.L., Polyak, I.K., and Häse, C.C. (2008) Re-emergence of Vibrio tubiashii in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. *Dis Aquat Organ* **82:** 119-134.

Erasmus, J.H., Cook, P.A., and Coyne, V.E. (1997) The role of bacteria in the digestion of seaweed by the abalone *Haliotis midae*. *Aquaculture* **155:** 377-386.

Erwin, P.M., Pineda, M.C., Webster, N., Turon, X., and Lopez-Legentil S. (2014) Down under the tunic: bacterial biodiversity hotspots and widespread ammonia-oxidizing archaea in coral reef ascidians. *ISME J* **8**: 575-588.

Erwin, P.M., and Thacker, R.W. (2008) Phototrophic nutrition and symbiont diversity of two Caribbean sponge–cyanobacteria symbioses. *Mar Ecol Prog Ser* **362:** 139-147.

Esteves, A.L., Hardoim, C.C., Xavier, J.R., Goncalves, J.M., and Costa, R. (2013) Molecular richness and biotechnological potential of bacteria cultured from *Irciniidae* sponges in the north-east Atlantic. *FEMS Microbiol Ecol* doi: 10.1111/1574-6941.12140.

Fan, L., Reynolds, D., Liu, M., Stark, M., Kjelleberg, S., Webster, N.S., *et al*. (2012) Functional equivalence and evolutionary convergence in complex communities of microbial sponge symbionts. *Proc Natl Acad Sci USA* **109:** E1878-E1887.

Faruque, S.M., Albert, M.J., and Mekalanos, J.J. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholera*. *Microbiol Mol Biol Rev* **62:** 1301-1314.

Fisher, C.R. (1990) Chemoautotrophic and methanotrophic symbiosis in marine invertebrates. *Rev Aquat Sci* **2:** 399-436.

Folmer, O., Black, M., Hoeh, W., Lutz, R., and Vrijenhoek, R. (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Mol Mar Biol Biotechnol* **3:** 294-299.

Fraune, S., and Bosch, T.C.G. (2007) Long-term maintenance of species-specific bacterial microbiota in the basal metazoan Hydra. *Proc Natl Acad Sci USA* **104:** 13146-13151.

Fraune, S., and Zimmer, M. (2008) Host-specificity of environmentally transmitted *Mycoplasma*-like isopod symbionts. *Environ Microbiol* **10:** 2497-2504.

Freitas, S., Hatosy, S., Fuhrman, J.A., Huse, S.M., Mark, D.B., Sogin, M.L., *et al*. (2102) Global distribution and diversity of marine *Verrucomicrobia*. *ISME J* **6:** 1499-1505.

Gallop. A., Bartrop, J., and Smith, D.C. (1980) The biology of chloroplast acquisition by *Elysia viridis*. *Proc R Soc B* **307:** 335-349.

Gao, J., and Hamann, M.T. (2011) Chemistry and biology of kahalalides. *Chem Rev* **111:** 3208-3235.

García-Rocha, M., Bonay, P., and Avila, J. (1996) The antitumoral compound Kahalalide F acts on cell lysosomes. *Cancer Lett* **99:** 43-50.

Gast, R.J., Moran, D.M., Dennett, M.R., and Caron, D.A. (2007) Kleptoplasty in an Antarctic dinflagellate: caught in evolutionary transition? *Environ Microbiol* **9:** 39-45.

Gavagnin, M., Marín, A., Mollo, E., Crispino, A., Villani, G., and Cimino, G. (1994) Secondary metabolites from Mediterranean *Elysioidea*: origin and biological role. *Comp Biochem Physiol* **108B***:* 107-115.

Gavagnin, M., Mollo, E., Castelluccio, F., Montanaro, D., Ortea, J., and Cimino, G. (1997) A novel dietary sesquiterpene from the marine sacoglossan *Tridachia crispata. Nat Prod Lett* **10:** 151-156.

Gavagnin, M., Mollo, E., Monatanaro, D., Ortea, J., and Cimino, G. (2000) Chemical studies of Caribbean sacoglossans: dietary relationships with green algae and ecological implications. *J Chem Ecol* **26:** 1563-1578.

Gavagnin, M., Spinella, A., Marín, A., Castelluccio, F., and Cimino, G. (1994) Polypropionates from the Mediterranean mollusk *Elysia timida. J Nat Prod* **57:** 298-304.

Göbbeler, K., and Klussmann-Kolb, A. (2011) Molecular phylogeny of the Euthyneura (Mollusca, Gastropoda) with special focus on Opisthobranchia as a framework for reconstruction of evolution of diet. *Thalassas* **27:** 121-154.

Golberg, K., Eltzov, E., Shnit-Orland, M., Marks, R.S., and Kushmaro, A. (2011) Characterization of quorum sensing signals in coral-associated bacteria. *Microb Ecol* **61:** 783-792.

Gomez-Gil, B., Soto-Rodríguez, S., García-Gasca, A., Roque, A., Vazquez-Juarez, R., Thompson, F.L., *et al*. (2004) Molecular identification of *Vibrio harveyi*-related isolates associated with diseased aquatic organisms. *Microbiol* **150:** 1769-1777.

Gil-Turnes, M.S., Hay, M.E., and Fenical, W. (1989) Symbiotic marine bacteria chemically defend crustacean embryos from a pathogenic fungus. *Science* **246:** 116-118.

Griffin, A.S., West, S.A., and Bucking, A. (2004) Cooperation and competition in pathogenic bacteria. *Nature* **430:** 1024-1027.

Hamann, M.T. (2004) Technology evaluation: Kahalalide F, PharmaMar. *Curr Opin Mol Ther* **6:** 657-665.

Hamann, M.T., Otto, C.S., and Scheuer, P.J. (1996) Kahalalides: bioactive peptides from a marine mollusk *Elysia rufescens* and its algal diet *Bryopsis* sp. *J Org Chem* **61:** 6594-6600.

Hamann, M.T., and Scheuer, P.J. (1993) Kahalalide F: a bioactive depsipeptide from the sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. *J Am Chem Soc* **115:** 5825-5826.

Händeler, K., Grzymbowski, Y.P., Krug, P.J., and Wägele, H. (2009) Functional chloroplasts in metazoan cells – unique evolutionary strategy in animal life. *Zool* **6:** 28 doi:10.1186/1742-9994-6-28.

Händeler, K., and Wägele, H. (2007) Preliminary study on molecular phylogeny of Sacoglossa and a compilation of their food organisms. *Bonn Zool Beitr* **55:** 231-254.

Händeler, K., Wägele, H., Wahrmund, U., Rüdinger, M., and Knoop, V. (2010) Slugs' last meals: molecular identification of sequestered chloroplasts from different algal origins in Sacoglossa (Opisthobranchia, Gastropoda). *Mol Ecol Resour* **10:** 968-978.

Hay, M.E., Pawlik, J.R., Duffy, E.J., and Fenical, W. (1989) Seaweed-herbivore-predator interactions: host-plant speciali- zation reduces predation on small herbivores. *Oecologia* **81:** 418-427.

Hentzer, M., Wu, H., Andersen, J.B., Riedel, K., Rasmussen, T.B., Bagge, N. *et al*. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* **22:** 3803-3815.

Hill, R. T. (2004) Microbes from marine sponges: a treasure trove of biodiversity for natural products discovery. In *Microbial diversity and bioprospecting.* A. T. Bull (ed.). ASM Press, Washington, D.C. p177-190.

Hill, R.T., Enticknap, J., Rao, K.V., and Hamann, M.T. (2003) Kahalalide-producing bacteria. Pub. No. US 2007/0196901 A1.

Hoffmiester, M., and Martin, W. (2003) Interspecific evolution: microbial symbionts, endosymbiosis and gene transfer. *Environ Microbiol* **5:** 641-649.

Holbro, T., Beerli, R.R., Maurer, F., Koziczak, M., Barbas, C.F., and Hynes, N.E. (2003) The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires

ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci USA* **100:** 8933-8938.

Hollants, J., Decleyre, H., Leliaert, F., De Clerck, O., and Willems, A. (2011a) Life without cell membrane: challenging the specificity of bacterial endophytes within *Bryopsis* (Bryopsidales, Chlorophyta). *BMC Microbiology* **11:** 255-265.

Hollants, J., Leliaert, F., Verbruggen, H., De Clerck, O., and Willems A. (2013) Host specificity and coevolution of Flavobacteriaceae endosymbionts within the siphonous green seaweed *Bryopsis*. *Mol Phylogenet Evol* **67:** 608-614.

Hollants, J., Leroux, O., Leliaert, F., Decleyre, H., De Clerck, O., and Willems, A. (2011b) Who is there? Exploration of endophytic bacteria within the siphonous green seaweed *Bryopsis* (Bryopsidales, Chlorophyta). *PLoS One* **6:** e26458.

Holmström, C., and Kjelleberg, S. (1994) The effect of external biological factors on settlement of marine invertebrate and new antifouling technology. *Biofouling* **8:** 147-160.

Hooper, C., Day, R., Slocombe, R., Handlinger, J., and Benkendorff K. (2007) Stress and immune responses in abalone: limitations in current knowledge and investigative methods based on other models. *Fish Shellfish Immunol* **22:** 363-379.

Huang, Z.B., Guo, F., Zhao, J., Li, W.D., and Ke, C.H. (2010) Molecular analysis of the intestinal bacterial flora in cage-cultured adult small abalone, *Haliotis diversicolor*. *Aquac Res* **41:** 760-769.

Huse, S.M., Welch, D.M., Morrison, H.G., and Sogin, M.L. (2010) Ironing out the wrinkles in the rare biosphere through improved OTU clustering. *Environ Microbiol* **12:** 1889-1898.

Ireland, C., and Faulkner, J (1981) The metabolites of the marine molluscs Tridachiella diomedea and Tridachia crispata. *Tetrahedron* **37:** 233-240.

Janmaat, M.L., Rodriguez, J.A., Jimeno, J., Kruyt, F.A., and Giaccone, G. (2005) Kahalalide F induces necrosis-like cell death that involves depletion of ErbB3 and inhibition of Akt signaling. *Mol Pharmacol* **68:** 502-510.

Janssen, P.H. (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Appl Environ Microbiol* **72:** 1719-1728.

Jensen, K.R. (1983) Factors affecting feeding selectivity in herbivorous Ascoglossa (Mollusca: Opisthobranchia). *J Exp Mar Biol Ecol* **66:** 135-148.

Jensen, K.R. (1993) Morphological adaptations and plasticity of radular teeth of the Sacoglossa (= Ascoglossa) (Mollusca: Opisthobranchia) in relation to their food plants. *Biol J Linn Soc* **48:** 135-155.

Jensen, K.R. (1994) Behavioural adaptations and diet specificity of sacoglossan opisthobranchs. *Ethol Ecol Evol* **6:** 87-101.

Jensen, K.R. (1996) Phylogenetic systematics and classification of the Sacoglossa (Mollusca, Gastropoda, Opisthobranchia). *Phil Trans R Soc Lond B* **351:** 91-122.

Jensen, K.R. (1997) Evolution of the Sacoglossa (Mollusca, Opisthobranchia) and the ecological associations with their food plants. *Evol Ecol* **11:** 301-335.

Jensen, K.R. (2007) Biogeography of the Sacoglossa (Mollusca, Opisthobranchia). *Bonn Zool Beitr* **55:** 255-281.

Kampfer, P., Thummes, K., Chu, H.I., Tan, C.C., Arun, A.B., Chen, W.M., *et al*. (2008) *Pseudacidovarox intermedius* gen. nov., sp. nov., a novel nitrogen-fixing betaproteobacterium isolated from soil. *Int J Syst Evol Microbiol* **58:** 491-495.

Kanda, A., Iwakoshi-Ukena, E., Takuwa-Kuroda, K., and Minakata, H. (2003) Isolation and characterization of novel tachykinins from the posterior salivary gland of the common octopus *Octupus vulgaris*. *Peptides* **24:** 35-43.

Kapareiko, D., Lim, H.J., Schott, E.J., Hanif, A., and Wikfors, G.H. (2011) Isolation and evaluation of new probiotic bacteria for use in shellfish hatcheries: II. Effects of a *Vibrio* sp. probiotic candidate upon survival of oyster larvae (*Crassostrea virginica*) in pilot-scale trials. *J Shellfish Res* **30:** 617-625.

Karim, M., Zhao, W., Rowley, D., Nelson, D., and Gomez-Chiarrii, M. (2013) Probiotic strains for shellfish aquaculture: protection of eastern oyster, *Crassostrea virginica*, larvae and juveniles against bacterial challenge. *J Shellfish Res* **32:** 401-408.

Kawaguti, S., and Baba, K. (1959) A preliminary note on a two-valved sacoglossan gastropod *Tamanovalva limax* n. gen., n. sp. from Tamano, Japan. *Biol J Okayama Univ* **5:** 177-184.

Kawaguti, S., and Yamasu, T. (1965) Electron microscopy on the symbiosis between an elysioid gastropod and chloroplasts from a green alga. *Biol J Okayama Univ* **2:** 57-64.

Kay, E.A. (1968) A review of the bivalved gastropods and a discussion of evolution within the Sacoglossa. *Symp Zool Soc Lond* **22:** 109-134.

Kelley, W.P., Wolters, A.M., Sack, J.T., Jockusch, R.A., Jurchen, J.C., Williams, E.R., *et al*. (2003) Characterization of a novel gastropod toxin (6-bromo-2-mercaptotryptamine) that inhibits shaker K channel activity. *J Biol Chem* **278:** 34934-34942.

Kembel, S.W., Eisen, J.A., Pollard, K.S., and Green, J.L. (2011) The phylogenetic diversity of metagenomes. *PLoS One* **6:** e23214. doi:10.1371/journal.pone.0023214.

Khan, S.T., Horiba, Y., Yamamoto, M., and Hiraishi, A. (2002) Members of the family *Comamonadaceae* as primary poly (3-hydroxybutyrate-co-3-hydoxyvalerate)-degrading denitrifiers in activiated sludge as revealed by a polyphasic approach. *Appl Environ Microbiol* **68:** 3206-3214.

King, G.M., Judd, C., Kuske, C.R., and Smith, C. (2012) Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS One* **7:** e51475. doi:10.1371/journal.pone.0051475.

Kirchman DL. **(**2002) The ecology of Cytophaga-Flavobacteria in aquatic environments. *FEMS Microbiol Ecol* **39:** 91-100.

Kuanpradit, C., Stewart, M.J., York, S.P., Degnan, B.M., Sobhon, P., Hanna, P.J., *et al*. (2012) Characterization of mucus-associated proteins from abalone (Haliotis) – candidates for chemical signaling. *FEBS J* **279:** 437-450.

Kurahashi, M. (2007) *Endozoicomonas elysicola* gen. nov., sp. nov., a gamma-proteobacterium isolated from the sea slug *Elysia ornata*. *Syst Appl Microbiol* **30:** 202-206.

Kurahashi, M., and Yokota, A. (2004) Agarivorans albus gen. nov., sp. nov., a gamma-proteobacterium isolated from marine animals *Int J Syst Evol Microbiol* **54:** 693-697.

Lane, D.J. (1991) 16S/23S rRNA sequencing, p 115-175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. John Wiley and Sons, New York.

Lokmer, A., and Wegner, K.M. (2014) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J* doi: 10.1038/ismej.2014.160.

Luber-Narod, J., Smith, B., Grant, W., Jimeno, J.M., López-Lázaro, L., Scotto, K., *et al*. (2000) In vitro safety toxicology of kahalalide F, a marine natural product with chemotherapeutic potential against selected solid tumors. *Clin Cancer Res* **6:** 4510S

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, *et al*. **(**2004) ARB: a software environment for sequence data. *Nucleic Acids Res* **32:** 1363-1371.

Maeda, T., Hirose, E., Chikaraishi, Y., Kawato, M., Takishita, K., Yoshida, T., *et al*. (2012) Algivore or phototroph? *Plakobranchus ocellatus* (Gastropoda) continuously acquires kleptoplasts and nutrition from multiple algal species in nature. *PLoS One* **7:** e42024.

Maeda, T., Kajita, T., Maruyama, T., and Hirano, Y. (2010) Molecular phylogeny of the Sacoglossa, with a discussion of gain and loss of kleptoplasty in the evolution of the group. *Biol Bull* **219:** 17-26.

Mao-Jones, J., Ritchie, K.B., Jones, L.E., and Ellner, S.P. (2010) How microbial community composition regulates coral disease development. *PLoS Biol* **8:** e1000345.

Mansson, M., Gram, L., and Larsen, T.O. (2011) Production of bioactive secondary metabolites by marine *Vibrionaceae*. *Mar Drugs* **9:** 1440-1468.

Marahiel, M.A., Stachelhaus, T., and Mootz, H.D. (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. *Chem Rev* **97:** 2651-2673.

Marín, A., Alvarez, L.A., Cimino, G., and Spinella A. **(**1991**)** Chemical defense in cephalaspidean gastropods: origin, anatomical location and ecological roles. *J Mollus Stud* **65:** 121-131.

Marín, A., and Ros. J. (2004) Chemical defenses in sacoglossan opisthobranchs: taxonomic trends and evolutive implications. *Sci. Mar.* **68:** 227-241.

Marín, A., and Ros, J.D. (1989) The chloroplast-animal association in four Iberian sacoglossan opisthobranchs: *Elysia timida*, *Elysia translucens*, *Thuridilla hopei* and *Bosellia mimetica*. *Sci Mar* **53:** 429-440.

Mayasich, S.A., and Smucker, R.A. (1987) Role of *Cristispira* sp. and other bacteria in the chitinase and chitobiase activities of the crystalline style of *Crassostrea virginica* (Gmelin). *Microb Ecol* **14:** 157-166.

McFarland, F.K., and Muller-Parker, G. (1993) Photosynthesis and retention of zooxanthellae and zoochlorellae within the aeolid nudibranch *Aeolidia papillosa*. *Biol Bull* **184:** 223-229.

McManus, G.B., Schoener, D.M., and Haberlandt, K. (2012) Chloroplast symbiosis in marine ciliate: ecophysiology and the risks and rewards of hosting foreign organelles. *Front Microbiol* **3:** 321 doi: 10.3389/fmicb.2012.00321.

Mearns-Spragg, A., Bregu, A., Boyd, K.G. and Burgess, J.G. (1998) Cross-species induction and enhancement of antimicrobial activity produced by epibiotic bacteria from marine algae and invertebrates, after exposure to terrestrial bacteria. *Lett Appl Microbiol* **27:** 142-146.

Méthot, P.O., and Alizon, S. What is a pathogen? Toward a process view of host-parasite interactions. *Virulence* **5:** 775-785.

Mikkelsen, P.M. (2002) Shelled Opisthobranchs. *Adv Mar Biol* **42:** 76-136.

Mohamed, N.M., Colman, A.S., Tal, Y., and Hill, R.T. (2008) Diversity and expression of nitrogen fixation genes in bacterial symbionts of marine sponges. *Environ Microbiol* **10:** 2910-2921.

Mohamed, N.M., Saito, K., Tal, Y., and Hill, R.T. (2010) Diversity of aerobic and anaerobic ammonia-oxidizing bacteria in marine sponges. *ISME J* **4:** 38-48.

Montalvo, N.F., and Hill, R.T. (2011) Sponge-associated bacteria are strictly maintained in two closely related but geographically distant sponge hosts. *Appl Environ Microbiol* **77:** 7207-7216.

Montalvo, N.F., Mohamed, N.M., Entichnap, J.J., and Hill, R.T. (2005) Novel *Actinobacteria* from marine sponges. *Antonie van Leeuwenhoek* **87:** 29-36.

Mozzachiodi, R., Scuri, R., Roberto, M., and Brunelli, M. (2001) Caulerpenyne, a toxin from the seaweed *Caulerpa taxifolia*, depresses after hyperpolarization in invertebrate neuron. *Neuroscience* **107:** 519-526.

Muscholl-Silberhorn, A., Thiel, V., and Imhoff, J.F. (2008) Abundance and bioactivity of cultured sponge-associated bacteria from the Mediterranean Sea. *Microb Ecol* **55:** 94-106.

Muyzer, G., DeWaal, E.C., and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59:** 695-700.

Neal, A.L., Ahmad, S., Gordon-Weeks, R., and Ton, J. (2012) Benzoxazinoids in root exudates of maize attract *Pseudomonas putida* to the rhizosphere. *PLoS One* **7:**e35498. doi:10.1371/journal.pone.0035498

Naidoo, K., Maneveldt, G., Ruck, K., and Bolton, J. (2006) A comparison of various seaweed-based diets and formulated feed growth rate of abalone in a land-based aquaculture system. *J Appl Phycol* **18:** 437-443.

Nyholm, S.V., Deplancke, B., Gaskins, H.R., Apicella, M.A., and McFall-Ngai, M.J. (2002) Roles of *Vibrio fischeri* and nonsymbiotic bacteria in the dynamics of mucus secretion furing symbiont colonization of the *Euprymna scolopes* light organ. *Appl Environ Microbiol* **68:** 5113-5122.

Nyholm, S.V., Stabb, E.V., Ruby, E.G., and McFall-Ngai, M.J. (2000) Establishment of an animal-bacterial association: recruiting symbiotic Vibrios from the environment. *Proc Natl Acad Sci USA* **97:** 10231-10235.

Ouwerkerk, J.P., de Vos, W.M., and Belzer, C. (2013) Glycobiome: bacteria and mucus at the epithelial interface. *Best Pract Res Clin Gastroenterol* **27:** 25-38.

Overbeek, R., Olson, R., Pusch, G.D., Olsen, G.J., Davis, J.J., Disz, T., *et al*. (2014) *The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res* **42:** D206-D214.doi: 10.1093/nar/gkt1226.

Paillard, C., Le Roux, F. and Borreg, J.J. (2004) Bacterial disease in marine bivalves, a review of recent studies: trends and evolution. *Aquat Living Resour* **17:** 477-498.

Pardy, R.L. (1980) Symbiotic algae and ^{14}C incorporation in the freshwater clam *Anodonta. Biol Bull* **158:** 349-355.

Pardy, R.L., and Lewin, R.A. (1981) Colonial ascidians with prochlorophyte symbionts: evidence from translocation of metabolites from alga to host. *Bull Mar Sci* **31:** 817-823.

Parsek, M.R., and Greenberg, E.P. (2005) Sociomicrobiology: the connections between quorum sensing and biofilms. *Trends Microbiol* **13:** 27-33.

Paul, V.J. and Fenical, W. (1986) Chemical defense in tropical green algae, order Caulerpales. *Mar Ecol Progr Ser* **34:** 157-169.

Paul, V.J., Arthur, K.E., Ritson-Williams, R., Ross, C., and Sharp, K. (2007) Chemical defenses: from compounds to communities. *Biol Bull* **213:** 226 -251.

Paul, V.J., and Van Alstyne, K.L. (1988) Use of ingested algal diterpenoid by *Elysia halimedae* Macnae (Opisthobranchia: Ascoglossa) as antipredator defenses. *J Exp Mar Biol Eco*l **119:** 15-29.

Pawlik, J.R. (1993) Marine invertebrate chemical defenses. *Chem Rev* **93:** 1911-1922.

Pawlik, J.R. (2011) The chemical ecology of sponges on Caribbean reefs: natural products shape natural systems. *Bio Sci* **61:** 888-898.

Pawlik, J.R., Albizati, K.F., and Faulkner, D.J. (1986) Evidence of a defensive role for limatulone, a novel triterpene from the interdial limpet *Collisella limatula*. *Mar Ecol Prog Ser* **30:** 251-260.

Pelletreau, K.N., Bhattacharya, D., Price, D.C., Worful, J.M., Moustafa, A., and Rumpho ME. 2011. Sea slug kleptoplasty and plastid maintenance in a metazoan. *Plant Physiol* **155:** 1561-1565.

Penesyan, A., Marshall-Jones, Z., Holmström, C., Kjelleberg, S., and Egan, S. (2009) Antimicrobial activity observed among cultured marine epiphytic bacteria reflects their potential as a source of new drugs. *FEM Microbiol Ecol* **69:** 113-124.

Phelan, R.W., O'Halloran, J.A., Kennedy, J., Morrissey, J.P., Dobson, A.D., O'Gara, F., *et al*. (2012) Diversity and bioactive potential of endospore-forming bacteria cultured from the marine sponge *Haliclona simulans*. *J Appl Microbiol* **112:** 65-78.

Piel, J. (2004) Metabolites from symbiotic bacteria. *Nat Prod Rep* **21:** 519-538.

Piel, J., Hui, D., Wen, G., Butzke, D., Platzer, M., Fusetani, N., *et al*. (2004) Antitumor polyketide biosynthesis by an uncultivated bacterial symbiont of the marine sponge *Theonella swinhoei*. *Proc Natl Acad Sci USA* **101:** 16222-16227.

Pierce, S.K., and Curtis, N.E. (2012) Cell biology of the chloroplast symbiosis in sacoglossan sea slugs. In International review of cell and molecular biology. K. W. Jeon (ed.). Academic Press, p123-148.

Pierce, S.K., Curtis, N.E., Massey, S.E., Bass, A.L., Karl, S.A., and Finney, C.M. (2006) A morphological and molecular comparison between *Elysia crispata* and a

new species of kleptoplastic sacoglossan sea slug (Gastropoda: Opisthobranchia) from the Florida Keys, USA. *Mollusc Res* **26:** 23-38.

Pierce, S.K., Fang, X., Schwartz, J.A., Jiang, X., Zhao, W., Curtis, N.E., *et al*. **(**2012) Transcriptomic evidence for the expression of horizontally transferred algal nuclear genes in the photosynthetic sea slug, *Elysia chlorotica*. *Mol Biol Evol* **29:** 1545-1556.

Pohnert, G., and Jung, V. (2003) Intracellular compartmentation in the biosynthesis of caulerpenyne; study on intact macroalgae using stable-isotope-labeled precursors. *Org Lett* **5:** 5091-5093.

Poretsky, R., Rodriguez, L.M., Luo, C., Tsementzi, D., and Konstantinidis, K.T. (2014) Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS One* **9:** e93827. doi:10.1371/ journal. pone.0093827.

Proksch, P., Edrada, R.A., and Ebel, R. **(**2002) Drugs from the sea – current status and microbiological implications. *Appl Microbiol Biotechnol* **59:** 25-34.

Pruzzo, C., Hug, A., Colwell, R.R., and Donelli, G. **(**2005) Pathogenic *Vibrio* species in the marine and estuarine environment, p 217-252. *In* Belkin S, Colwell RR (ed), Oceans and health: pathogens in the marine environment, Springer, New York, NY.

Rappé, M.S., and Giovannoni, S.J. (2003) The uncultured microbial majority. *Annu Rev Microbiol* **57:** 369-394.

Ritchie, K.B. (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar Ecol Prog Ser* **322:** 1-14.

Roeselers, G., and Newton, L.G. (2012) On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Appl Microbiol Biotechnol* **94:** 1-10.

Roeselers, G., Mittge E.K., Stephens, W.Z., Parichy, D.M., Cavanaugh, C.M., Guillemin, K., et al. (2011) Evidence for a core gut microbiota in the zebrafish. *ISME J* **5:** 1595-1608.

Rosenberg, E., Koren, O., Reshef, L,. Efrony, R., and Zilber-Rosenberg, I. (2007) The role of microorganisms in coral health, disease and evolution. *Nat Rev Microbiol* **5:** 355-362.

Rumpho, M.E. (2011) Sea slug kleptoplasty and plastid maintenance in a metazoan. *Plant Physiol* **155:** 1561-1565.

Rumpho, M.E., Pelletreau, K.N., Moustafa, A., and Bhattacharya, D. (2011) The making of a photosynthetic animal. *J Exp Biol* **214:** 303-311.

Rumpho, M.E., Mujer, C.V., Andrews, D.L., Manhart, J.R., and Pierce, S.K. 1994. Extraction of DNA from mucilaginous tissues of a sea slug (*Elysia chlorotica*). *Biotech* **17:** 1097-1101.

Rumpho, M.E., Summer, E.J., Green, B.J., Fox, T.C., and Manhart, J.R. (2001) Mollusc/algal chloroplast symbiosis: how can isolated chloroplasts continue to

function for months in the cytosol of a sea slug in the absence of an algal nucleus? *Zoology* **104:** 303-312.

Rumpho, M.E., Summer, E.J., and Manhart, J.R. (2000) Solar-powered sea slugs. Mollusc/algal chloroplast symbiosis. *Plant Physiol* **123:** 29-38.

Ruby, E.G. (1996) Lessons from a cooperative, bacterial-animal association. The *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. *Annu Rev Microbiol* **50:** 591-624.

Sacristan-Soriano, O., Banaigs, B., Casamayor, E.O., and Becerro, M.A. (2011) Exploring the links between natural products and bacterial assemblages in the sponge *Aplysina aerophoba*. *Appl Environ Microbiol* **77:** 862-870.

Sadaie, T., Sadaie, A., Takada, M., Hamano, K., Ohnishi, J., Ohta, N., *et al*. (2007) Reducing sludge production and the domination of *Comamonadaceae* by reducing the oxygen supply in the wastewater treatment procedure of a food-processing factory. *Biosci Biotechnol Biochem* **71:** 791-799.

Saitou, N., and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4:** 406-425.

Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., *et al*. (2009) Introducing mothur: open-source, platform-independent, community-

supported software for describing and comparing microbial communities. *Appl Environ Microbiol* **75:** 7537-7541.

Schmidt, E.W., Nelson, J.T., Rasko, D.A., Sudek, S., Eisen, J.A., Haygood, M.G., *et al*. (2005) Patellamide A and C biosynthesis by a microcin-like pathway in *Prochloron didemni*, the cyanobacterial symbiont of *Lissoclinum patella*. *Proc Natl Acad Sci USA* **102:** 7315-7320.

Serova, M., de Gramont, A., Bieche, I., Riveiro, M.E., Galmarini, C.M., Aracil, M., *et al*. (2013) Predictive factors of sensitivity of Elisidepsin, a novel Kahalalide F-derived marine compound. *Mar Drugs* **11:** 994-959.

Sewell, J.M., Mayer, I., Langdon, S.P., Smyth, J.F., Jodrell, D.I., and Guichard, S.M. (2005) The mechanism of action of Kahalalide F: variable cell permeability in human hepatoma cell lines. *Eur J Cancer* **41:** 1637-1644.

Sharp, K.H., Ritchie, K.B., Schupp, P.J., Ritson-Williams, R., and Paul, V.J. (2010) Bacterial acquisition in juveniles of several broadcast spawning coral species. *PLoS One* **5:** e10898.

Simmons, T.L, Coates, R.C., Clark, B.R., Engene, N., Gonzalez, D., Esquenazi, E., *et al***.** (2008) Biosynthetic origin of natural products isolated from marine microorganisms-invertebrate assemblages. *Proc Natl Acad Sci USA* **105:** 4587-4594.

Slater, J.H., and Bull, A.T. (1982) Environmental microbiology: biodegradation. *Philos Trans R Soc Lond B Biol Sci* **297:** 575-597.

Sminia, T., and Van der Knaap, W.P.W. (1986) Immunorecognition in invertebrates with special reference to mollusks, p 112-124. *In* Brehelin M (ed), Immunity in invertebrates. Springer-Verlag, Berlin.

Smyrniotopoulos, V., Abatis, D., Tziveleka, L.A., Tsitsimpikou, C., Roussis, V., Loukis, A., *et al*. **(**2003) Acetylene sesquiterpenoid esters from the green alga *Caulerpa prolifera*. *J Nat Prod* **66:** 21-24.

Somerville, C.C., Knight, I.T., Straube, W.L., and Colwell, R.R. (1989) Simple, rapid method for direct isolation of nucleic acids from aquatic environments. *Appl Environ Microbiol* **55:** 548-554.

Steneck, R.S., and Watling, L. (1982). Feeding capabilities and limitation of herbivorous molluscs: A functional group approach. *Marine Biol* **68:** 299-319.

Stevenson, B.S., Eichorst, S.A., Wertz, J.T., Schmidt, T.M., and Breznak, J.A. (2004) New strategies for cultivation and detection of previously uncultured microbes. *Appl Environ Microbiol* **70:** 4748 -4755.

Suarez-Jimenez, G.M., Burgos-Hernandez, A., and Ezquerra-Brauer, J.M. **(**2012) Bioactive peptides and depsipeptides with anticancer potential: sources from marine animals. *Mar Drugs* **10:** 963-986.

Tait, K., Williams, P., Cámara, M., Williamson, H., Gan Chan, K., and Joint, I. (2009) Quorum sensing signal molecule synthesis and turnover in polymicrobial marine biofilms modulates communication with algal zoospores. *Environ Microbiol* **11:** 1792-1802.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013) MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* **30:** 2725-2729.

Taylor, J.D., and Glover, E.A. (2010) Chemosymbiotic bivalves, p 107-135. In Kiel S (ed), Topics in Geobiology. Springer, Netherlands.

Taylor, M.W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: evolution, ecology, and biotechnological potential. *Microbiol Mol Biol Rev* **71:** 295-347.

Thomas, F., Hehemann, J.H., Rebuffet, E., Czizek, M., and Michel G. **(**2011) Environmental and gut Bacteroidetes: the food connection. *Front Microbiol* **2:**93 doi: 10.3389/fmicb.2011.00093.

Thompson, F.L., Gevers, D., Thompson, C.C., Dawyndt, P., Naser, S., Hoste, B. *et al*. (2005) Phylogeny and molecular identification of Vibrios on the basis of multilocus sequence analysis. *Appl Environ Microbiol* **71:** 5107-5115.

Thompson, F.L., Iida, T., and Swings, J. (2004) Biodiversity of Vibrios. *Microbiol Mol Biol Rev* **68:** 403-431.

Thompson, T.E. (1976). Biology of Opisthobranch Molluscs, Volume 1, Ray Society, London.

Thompson, T.E., and Brown GH (1984). Biology of Opisthobranch Molluscs. Volume 1I, Ray Society, London.

Trench, R.K. (1975) Of leaves that crawl: functional chloroplast in animal cells. *Symp Soc Exp Biol* **29:** 229-265.

Uchimura, M., Sandeauz, R., and Larroque, C. (1999) The enzymatic detoxifying system of a native Mediterranean scorpion fish is affected by *Caulerpa toxifolia* in its environment. *Environ Sci Technol* **33:** 1671-1674.

Urakawa, H., Dubilier, N., Fujiwara, Y., Cunningham, D.E., Kojima, S., Stahl, D.A. (2005) Hydrothermal vent gastropods from the same family (Provannidae) harbour ε – and γ – proteobacterial endosymbionts. *Environ Microbiol* **7:** 750-754.

van Passel, M.W.J., Kant, R., Zoetendal, E.G., Plugge, C.M., Derrien, M., Malfatti, S.A., *et al*. (2011) The genome of Akkermansia muciniphila, a dedicated intestinal mucin degrader, and its using in exploring intersinal metagenomes. *PLoS One* **6:** e16876. doi:10.1371/journal.pone.0016876

Verdugo, P., Deyrup-Olsen, I., Aitken, M., Villalon, M., and Johnson, D. (1987) Molecular mechanism of mucin secretion: the role of intragranular charge shielding. *J*

Dent Res **66:** 506-508.

Venn, A.A., Loram, J.E., and Dougalas, A.E. (2008) Photosynthetic symbiosis in animals. *J Exp Bot* **59:** 1069-1080.

Wägele, H. (2004) Potential key characters in opisthobranchia (Gastropoda, Mollusca) enhancing adaptive radiation. *Org Div Evol* **4:** 175-188.

Wägele, H., Deutsch, O., Händeler, K., Martin, R., Schmitt, V., Christa, G., *et al*. (2011) Transcriptomic evidence that longevity of acquired plastids in the photosynthetic slugs *Elysia timida* and *Plakobranchus ocellatus* does not entail lateral transfer of algal nuclear genes. *Mol Biol Evol* **28:** 699-706.

Wägele, H., and Klussmann-Kolb (2005) Opisthobranchia (Mollusca, Gastropoda) - more than just slimy slugs. Shell reduction and its implications on defence and foraging *Front Zool* **2:** 3.doi:10.1186/1742-9994-2-3.

Wahl, M., Jensen, P.R., and William, F. (1994) Chemical control of bacterial epibiosis on ascidians. *Mar Ecol Prog Ser* **110:** 45-57.

Waksman, S.A., Carey, C.L., and Reuszer, H.W. (1933) Marine bacteria and their role in the cycle of life in the sea decomposition of marine plant and animal residues by bacteria. *Biol Bull* **65:** 57-79.

Wang, S., Huang, X., Lee, C.K., and Liu, B. (2010) Elevated expression of erbB3 confers paclitaxel resistance in erbB2-overexpressing breast cancer cells via upregulation of Survivin. *Oncogene* **29:** 4225-4236.

Waugh, G.R., and Clark, K.B. (1986) Seasonal and geographic variation in chlorophyll level of *Elysia tuca* (Ascoglossa, Opisthobranchia). *Mar Biol* **92:** 483-487.

Wertz, J.T, Kim, E., Breznak, J.A., Schmidt, T.M., and Rodrigues, J.L. (2012) Genomic and physiological characterization of the *Verrucomicrobia* isolate *Diplosphaera colitermitum* gen. nov., sp. nov., reveals microaerophily and nitrogen fixation genes. *Appl Environ Microbiol* **78:** 1544-1555.

Whithear, K. (2001) Diseases due to mycoplasmas, p 413-422. *In* Williams ES, Barker IK (ed), Infectious diseases of wild mammals. Iowa University Press, Ames, Iowa.

Wiese, J., Thiel, V., Nagel, K., Staufenberger, T., and Imhoff, J.F. (2009) Diversity of antibiotic active bacteria associated with the brown alga *Laminaria saccharina* from the Baltic Sea. *Mar Biotechnol* **11:** 287-300.

Wietz, M., Mansson, M., Gotfredsen, C.H., Larsen, T.O., and Gram, L. (2010) Antibacterial compounds from marine *Vibrionaceae* isolated on a global expedition. *Mar Drugs* **8:** 2946-2960.

Williams, P., Winzer, K., Chan, W.C., and Cámara, M. (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* **362:** 1119-1134.

Williams, S.I., and Walker, D.I. (1999) Mesoherbivore-macroalgal interactions: Feeding ecology of sacoglossan sea slugs (Mollusca, Opisthobranchia) and their effects on their food algae. *Mar Biol Annu Rev* **37:** 87-128.

Wisniewski-Dye, F., and Downie, J.A. (2003) Quorum sensing in *Rhizobium*. *Antonie Van Leeuwenhoek* **81:** 397-407.

Yoon, J., Matsuo, Y., Matsuda, S., Adachi, K., Kasai, H., and Yokota, A. (2007) *Rubritalea spongiae* sp. nov. and *Rubritalea tangerina* sp. nov., two carotenoid- and squalene-producing marine bacteria of the family *Verrucomicrobiaceae* within the phylum '*Verrucomicrobia*', isolated from marine animals. *Int J Syst Evol Microbiol* **57:** 2337-2343.

Young, B.C., Golubchick, T., Batty, E.M., Fung, R., Larner-Svensson, H., Votintseva, A.A., et al. (2012) Evolutionary dynamics of Staphylococcus aureus during progression from carriage to disease. *Proc Natl Acad Sci USA* **109:** 4550-4555.

Yue, J.C., and Clayton, M.K. (2005) A similarity measure based on species proportions. *Commun Stat Theo Methods* **34:** 2123-2131.

Zan, J., Cicirell, E.M., Mohamed, N.M., Sibhatu, H., Kroll, S, Choi, O., *et al*. (2012) A complex LuxR-LuxI type quorum sensing network in a rosebacterial marine sponge symbiont activates flagellar motility and inhibits biofilm formation. *Mol Microbiol* **5:** 916-933.

Zatylny, C., Gagnon, J., Boucaud-Camou, E., and Henry, J. (2000). ILME: a waterbourne pheromonal peptide released by the eggs of *Sepia officinalis*. *Biochem Biophys Res Commun* **275:** 217-222.

Zhang, D., Xu, Z., Sun, W., and Karaolis, D.K.R. (2003) The *Vibrio* pathogenicity island-encoded mop protein modulates the pathogenesis and reactogenicity of epidemic *Vibrio cholerae. Infect Immun* **71:** 510-15.

Zhang, F., Pita, L., Erwin, P.M., Abaid, S., Lopez-Legentil, S., and Hill, R.T. (2014) Symbiotic archaea in marine sponges show stability and host specificity in community structure and ammonia oxidation functionality. *FEMS Microbiol Ecol* doi: 10.1111/1574-6941.12427.

Zheng, L., Han, X., Chen, H., Lin, W., and Yan, X. (2005) Marine bacteria associated with the marine macroorganisms: the potential antimicrobial resources. *Annuals Microbiol* **55:** 119-124.

Zhu, J., Chai, Y., Zhong, Z., Li, S., and Winans, S.C. (2003) Agrobacterium bioassay strain for ultrasensitive detection of *N*-acylhomoserine lactone-type quorum-sensing

molecules: detection of autoinducers in *Mesorhizobium huakuii*. *Appl Environ Microbiol* **69:** 6949-6953.

Zimmer, B.L., May, A.L., Bhedi, C.D., Dearth, S.P., Prevatte, C.W., Pratte, Z., *et al*. (2014) Quorum sensing signal production and microbial interactions in a polymicrobial disease of corals and the coral surface mucopolysaccharide layer. *PLoS One* **9:** e108541. doi:10.1371/journal.pone.0108541.